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(54) Title: PROGESTIN-REGULATED GENE

(57) Abstract

Isolated DNA molecules are disclosed corresponding to a novel progestin-regulated gene (PRG1). The PRG1 polypeptide (PRG1) and uses thereof are also described, and include assays for assessing progestin-responsiveness in a subject.

### PROGESTIN-REGULATED GENE

This invention relates to a novel progestin-regulated gene. The protein or polypeptide encoded by this gene appears to have a novel enzymatic activity that may be useful as a readily detectable marker for progestin-responsiveness.

The sex steroid hormone progesterone has two major roles in mammalian physiology. First, progesterone is involved in preparing the uterus for implantation of the fertilized ovum. Second, progestins have proliferative and differentiating effects on mammary epithelium (1, 2). Progesterone is essential for lobuloalveolar development and preparation for lactation: when ovulation is established progesterone, produced by the corpus luteum, stimulates growth of the lobuloalveolar structures and during pregnancy promotes branching of the ductal system and differentiation of alveolar cells into secretory cells ready for milk production. The importance of progestin in these processes is clearly illustrated in progesterone receptor (PR) knockout mice, which fail to develop lobuloalveolar structures (3). Progestins may also have a role in regulating cell proliferation in the human breast. Mitotic activity in breast epithelium varies in a cyclic manner through the menstrual cycle and a role for progesterone in this process is suggested by observations that levels of this hormone and epithelial cell proliferation are both maximal during the late secretory phase (4). Some breast tumours retain progesterone responsiveness and the use of high doses of synthetic progestins are recognised endocrine therapies for PR-positive breast cancers, since in this scenario progestins have an antiproliferative effect (5). Progestins also have predominantly growth inhibitory effects on human breast cancer cell lines *in vitro*, although under certain conditions they may stimulate growth (2, 6 and references therein). Mechanistic studies have clearly defined both a stimulatory and inhibitory effect of progestins on breast cancer cell cycle progression (6) but the functional consequences of these effects *in vivo* remain to be defined. This is of considerable importance given the wide spread pharmacological usage of progestins in oral contraceptives and in hormone replacement therapy.

The mechanisms underlying the biological effects of progestins in the normal breast and in breast cancer are only partially understood. Progestin action is mediated primarily via the PR, which upon activation by ligand binding interacts with gene promoter sequences containing progesterone

responsive elements (PREs) to regulate gene transcription. Very few mammalian genes have been described that are directly regulated by progestins in this manner; examples include *c-jun* (7), *c-fos* (8), fatty acid synthetase (FAS) (9), PR (10, 11), and uteroglobin (12, 13). While progestin action ultimately involves changes in the levels of large numbers of mRNAs and proteins, many of these require intermediary *de novo* protein synthesis. Furthermore, specific genes that mediate the proliferative effects of progestins are likewise poorly defined. Thus much remains to be learned about genes induced as an acute response to progestin treatment and their role in mediating progestin effects on cell proliferation and differentiation.

Several known progestin-regulated genes can be classed as those whose functions are important in differentiation effects mediated by progestin. Examples include FAS (9), alkaline phosphatase (14) and lactate dehydrogenase (15). While these are probably not involved in the proliferative effects of progestin a number of progestin-related genes related to steroid and growth factor action might contribute to these effects at least indirectly. Examples include estrogen receptor (16), PR (11), retinoic acid receptors (17), epidermal growth factor receptor (6, 18), prolactin receptor (19), insulin-like growth factors  $\alpha$  and  $\beta$ 1 (6, 8, 23, 24), 17 $\beta$ -hydroxysteroid dehydrogenase (25) and insulin-like growth factor binding proteins 4 and 5 (26). However, evidence that these gene products are direct mediators of the stimulatory and inhibitory effects of progestins remains to be determined. Potentially of more interest are progestin-regulated genes with known roles in cell cycle control i.e. *c-myc*, *c-fos* (6, 8), *c-jun* (7) and cyclin D1 (27). Progestin induction of *c-myc* and cyclin D1 is closely related to changes in cell cycle progression (6, 27). While the timing of the induction of *c-myc* mRNA, evident after 30 min. of progestin treatment, suggests a potential direct effect of progestins, the slower induction of cyclin D1 mRNA which is maximal at 6 hours could result from the prior induction of other genes that are the primary and specific targets of progestins. Given the established central role for cyclin D1 in steroid and growth factor regulation of breast cancer cell cycle progression (27-30) identification of progestin regulated genes which control cyclin D1 gene expression might link progestin action to the cell cycle.

Using serum-free conditions, studies in this laboratory have shown that T-47D human breast cancer cells which were stimulated to grow with

insulin, undergo a transient increase in cell cycle progression in response to progestins, with an increased rate of progression through G1 and a transient increase in the S-phase population. These cells complete a round of replication and thereafter become growth arrested early in G1 phase.

5           The present invention arose out of a study using this model system to identify novel progestin regulated genes involved in early cell cycle stimulatory responses to progestin or other aspects of progestin action in human breast cancer cells. RNA extracted from T-47D cells grown under serum-free conditions and treated with the synthetic progestin ORG 2058 for  
10       3 hours was used as the template for cDNA synthesis and analysis by the differential display technique (mRNA fingerprinting) (31). Several candidate PCR fragments were identified by this method and characterisation by sequence and Northern analysis of some of these led to the identification and characterisation of a clone, PRG1, which appears to represent a novel  
15       progestin-regulated gene.

Thus, in a first aspect, the present invention provides an isolated DNA molecule comprising a nucleotide sequence substantially corresponding or, at least, >80% (more preferably, >90%) homologous to any one of the nucleotide sequences shown at:

- 20       (i)       Figure 2B from nucleotide 1 to 3018;  
         (ii)       Figure 2B from nucleotide 1 to 470;  
         (iii)      Figure 2B from nucleotide 141 to 3018; and  
         (iv)      Figure 2B from nucleotide 470 to 2103.

Preferably, the isolated DNA molecule is of human origin. More  
25       preferably, the isolated DNA molecule is of human kidney cell or breast cancer cell origin, and/or encodes a protein normally expressed in human kidney cells, breast tissue or tumour cells.

The isolated DNA molecule may be incorporated into plasmids or expression vectors, which may then be introduced into suitable bacterial,  
30       yeast and mammalian host cells. Such host cells may be used to express the polypeptide encoded by the isolated DNA molecule.

The predicted amino acid sequence of the polypeptide encoded by PRG1 shows substantial homology (~70%) with human liver 6-phosphofructo-2-kinase/fructose 2,6 biphosphatase (PFK-2/FBPase-2) and it  
35       is postulated that the protein encoded by PRG1 may have activities similar to this bifunctional enzyme.

Thus, in a second aspect, the present invention provides a polypeptide in a substantially pure form, said polypeptide comprising an amino acid sequence substantially corresponding to that shown at Figure 2B or an enzymatic portion thereof.

5 Preferably, the polypeptide of the second aspect is full length.

The polypeptide of the second aspect may be used to raise monoclonal or polyclonal antibodies which may be used, for example, in affinity purification processes or in various ELISA type assays.

10 Thus, in a third aspect, the present invention provides an antibody specific to the polypeptide of the second aspect.

As will be seen hereinafter, PRG1 appears to be directly regulated by progestin. PRG1 may, therefore, provide a useful marker for progestin-responsiveness in a subject. For example, as a marker of breast tumour responsiveness to progestins - i.e. high levels may indicate that the tumour is responsive to progestins and could be sensitive to progestin therapy. PRG1 may also be a useful prognostic marker since hormonally responsive tumours often have a better prognosis (i.e. patients have longer disease-free survival and overall survival). Thus, levels of PRG1 mRNA present in isolated cells or tissue samples may be assessed by DNA or RNA probes or primers in hybridisation assays or PCR analysis. Suitable probes and primers, which are preferably of a length greater than 10 nucleotides, are to be considered as forming part of the present invention. Alternatively, the level of PRG1 polypeptide may be assessed through the use of the abovementioned antibodies. However, the postulated enzymatic activity of the PRG1 polypeptide provides the potential for a more convenient assay wherein the level of PRG1 polypeptide would be determined by assessment of enzyme activity.

Thus, in a fourth aspect, the present invention provides as assay for assessing progestin-responsiveness in a subject comprising the steps of;

- 30 (i) isolating cells or tissue from said subject; and  
(ii) detecting the presence of a polypeptide comprising an amino acid sequence substantially corresponding to that shown at Figure 2B.

Preferably, the polypeptide detection step involves providing a substrate for said polypeptide, said substrate normally converted by said polypeptide to a readily detected product.

35

In some circumstances, it may be preferred to expose the isolated cells or tissue to progestin or an agonist compound and, subsequently, determine whether the progestin or agonist compound has induced the production of PRG1 polypeptide.

5           The postulated enzyme activity of the PRG1 polypeptide also suggests that this polypeptide has an involvement in cell cycle (growth) regulation and is likely to be involved in control of glycolytic/gluconeogenic/lipogenic pathways not only in progestin target tissues but in a wide range of different tissue types. Indeed, since PRG1  
10       appears to be expressed in tissues with a probable low fraction of proliferating cells it is unlikely that the function of the PRG1 polypeptide is restricted to growth regulation. More likely, PRG1 is more generally involved in glycolytic/gluconeogenic/lipogenic control. This may be of particular significance as the other related human enzyme PFK-2/FBPase-2, which has  
15       an established important role in glycolytic control, has only a limited tissue distribution (e.g. liver). Thus, the administration of PRG1 polypeptide may be of significant therapeutic value particularly for treatment of diabetes, obesity or other disorders of energy metabolism. Therapeutic amounts are likely to be similar to normal endogenous levels (which will vary from tissue  
20       to tissue) or may be at significantly higher levels, depending on the level and type of activity desired.

          Alternatively, the enzyme activity of the PRG1 polypeptide could be regulated by pharmacological means for the treatment of proliferative disorders, such as malignant or non-malignant hyperproliferative disease (e.g.  
25       breast and other cancers, and dermatological diseases). Further, administration of PRG1 polypeptide may be of therapeutic value in the control of reproductive function.

          More specifically, the enzyme activity of the PRG1 polypeptide could be regulated by;  
30       synthetic compounds, either stimulatory or inhibitory (i.e. agonists or antagonists),  
          ribozymes specific for PRG1 (i.e. to down-regulate endogenous PRG1 activity), and  
          gene therapy using expression vectors or oligonucleotides or other  
35       delivery systems (e.g. viral) containing a nucleotide sequence encoding PRG1

sense (i.e. to augment endogenous PRG1 polypeptide levels and activity) or antisense (i.e. to down-regulate endogenous PRG1 levels and activity).

Agonist or antagonist compounds could be identified by their ability to inhibit/stimulate the enzyme activity of PRG1 polypeptide. For example, screening assays could be conducted to identify compounds that modulate 6-phosphofructo-2-kinase activity by measuring the rate of production of fructose-2,6-biphosphate from fructose-6-phosphate (assaying fructose-2,6-biphosphate by its ability to activate pyrophosphate-dependent 6-phosphofructo-1-kinase from potato tubers) (58). Alternatively, screening assays could be conducted to identify compounds that modulate fructose-2,6-biphosphatase activity by measuring [ $^{32}\text{P}$ ] release from [ $2\text{-}^{32}\text{P}$ ] fructose-2,6-bisphosphate (59). Such screening assays may be performed using *in vitro* systems such as dissolved pure PRG1 polypeptide or a whole cell lysate of cells expressing PRG1.

Such agonist and antagonist compounds may include compounds which influence enzymatic activity by a number of mechanisms such as the alteration of substrates to the enzyme's active site(s), either by acting as alternative substrates or by binding to PRG1 polypeptide to alter its structure, or by influencing processes involved in the activation of the PRG1 polypeptide (e.g. phosphorylation of regulatory domains).

Results provided hereinafter strongly suggest that induction of PRG1 by progestin is a direct transcriptional effect of ligand-activated PR on a putative progestin-regulatory element(s) (PRE) located within the PRG1 gene.

Thus, in a fifth aspect, the present invention provides an isolated DNA molecule comprising a progestin-regulatory element (PRE) derived from a DNA molecule according to the first aspect of the invention.

The DNA molecule of the fifth aspect may be used as a controlling element for PRG1 (and potentially for other genes containing similar promoter elements), for novel therapeutics to control gene expression or could be utilised in DNA constructs designed to express RNA/protein sequences in response to progestins (e.g. progestin-inducible plasmids) which could be useful as research tools for studying gene expression in cell lines. The DNA molecule of the fifth aspect could also be of use in gene therapy directed to progestin-responsive tissues e.g. breast, uterus.

The term "substantially corresponds" as used herein in relation to the nucleotide sequence is intended to encompass minor variations in the

nucleotide sequence which due to degeneracy in the DNA code do not result in a change in the encoded protein. Further, this term is intended to encompass other minor variations in the sequence which may be required to enhance expression in a particular system but in which the variations do not result in a decrease in biological activity of the encoded protein.

The term "substantially corresponding" as used herein in relation to amino acid sequence is intended to encompass minor variations in the amino acid sequence which do not result in a decrease in biological activity of the encoded protein. These variations may include conservative amino acid substitutions. The substitutions envisaged are:-

G,A,V,I,L,M; D, E; N,Q; S,T; K,R,H; F,Y,W,H; and P,N $\alpha$ -alkalamino acids.

The invention will now be further described with reference to the following non-limiting example and accompanying figures.

#### **Brief Description of the Figures:**

**Figure 1: Identification of differentially expressed cDNAs in T-47D cells treated with the synthetic progestin ORG 2058.**

A) Identification of PIG1 by differential display. Total RNA obtained from T-47D cells treated with ORG 2058 or ethanol for 3h. was used as a template for differential display PCR reactions with 5'-T<sub>12</sub>GG and 5'-CAAACGTCGG as primers. The PCR products were separated on a 6% polyacrylamide denaturing gel and the gel exposed to x-ray film. The arrows indicate PCR products present at a higher level in progestin treated (T) compared with ethanol control (C).

B) Confirmation of the progestin induction of PIG1 by Northern analysis. T-47D cells proliferating in insulin-supplemented serum-free medium were treated with 10nM ORG 2058 (T) or ethanol vehicle (C) for 3h and total RNA was harvested for Northern analysis. The Northern blot was probed with the PIG1 fragment.

**Figure 2: Determination of the PRG1 cDNA sequence.**

A) A schematic representation of PRG1 structure with a restriction map for the PRG1 cDNA and the cDNA clones used to derive the PRG1 sequence



shown beneath. The initial PCR cDNA fragment identified by differential display was designated PIG1. All the cDNA clones were isolated from a human kidney cDNA library with the exception of H7.1 which was isolated from a human heart cDNA library. Clone 19.1 is a chimeric clone. The cosmid clone containing genomic sequence, part of which overlaps with PRG1 cDNA sequence, was obtained from the Genbank database and is shown above the PRG1 sequence. The numbers refer to distances in nucleotides.

B) Nucleotide and deduced amino acid sequence of PRG1. The nucleotide sequence determined from cDNA clones is shown in uppercase lettering whereas the nucleotide sequence obtained from the cosmid genomic clone CR1-JC2015 is shown in lowercase lettering. The translation termination codon is shown by an asterisk in the amino acid sequence. The in-frame termination codon that precedes the initiating methionine is underlined. The numbers refer to distances in nucleotides.

**Figure 3: Amino acid sequence homologies of human and bovine PFK-2/FBPase-2 isoforms.**

Amino acid sequence homology between PRG1 and PFK-2/FBPase-2 isoforms from human liver, bovine brain and bovine heart. An alignment of the amino acid sequences was obtained using the computer programs MacVector™ 4.5.3 and SeqVu. Identical residues found in three or four of the polypeptides are boxed. Amino acids are numbered from the first residue. The bovine brain sequence is believed to be incomplete (39). Bov., bovine; hum., human.

**Figure 4: Expression of PRG1 mRNA in different human tissues and breast cancer and normal breast cell lines.**

A) Northern blot analysis of total RNA from different human breast cancer and normal breast cell lines. The blot was probed with a 1.8 kb cDNA sub-clone of PRG1 and an oligonucleotide complementary to 18S rRNA as a loading control.

B) Northern blot analysis of poly A<sup>+</sup> RNA from human tissues. The blot was hybridized with a 1.8 kb cDNA sub-clone of PRG1. Molecular sizes of markers are indicated. PBL, peripheral blood leukocytes.

**Figure 5: Regulation of PRG1 mRNA expression by the synthetic progestin ORG 2058.**

5 T-47D cells proliferating in insulin-supplemented serum-free medium were treated with 10 nM ORG 2058 (closed circles) or ethanol vehicle (open circles) and total RNA was harvested for Northern analysis. The Northern blot shown in panel A was probed with a 1.8 kb cDNA sub-clone of PRG1 and with an oligonucleotide complementary to 18S rRNA as a control for loading.  
10 Positions of the 28S and 18S ribosomal bands are indicated. Values in panel B were obtained by densitometric analysis of the autoradiograph and are expressed relative to control at 0 h.

15 **Figure 6: Regulation of PRG1 mRNA expression in MCF-7 and MDA-MB-231 cells by ORG 2058 and dexamethasone.**

MCF-7 (PR +ve, GR +ve) and MDA-MB-231 (PR -ve, GR +ve) cells proliferating in RPMI 1640 medium supplemented with 5% fetal calf serum were treated with ORG 2058 (10 nM), dexamethasone (100 nM) or ethanol vehicle and harvested for Northern analysis at 3 h. and 6 h. Densitometric analysis of the Northern blot hybridised with 1.8 kb cDNA sub-clone of PRG1 is presented expressed relative to control at 0 h. and adjusted for loading.  
20 The data in Panel B were obtained from the mean of two experiments.

25 **Figure 7: Antagonism of ORG 2058 induction of PRG1 mRNA by the antiprogestin RU 486.**

A) T-47D cells proliferating in insulin-supplemented serum-free medium were treated with ORG 2058 (10 nM), RU 486 (100 nM), the two compounds simultaneously (ORG 2058 + RU 486) or ethanol vehicle and harvested for Northern analysis at 3 h. The Northern blot was probed with the PRG1 fragment of PRG1 and with an oligonucleotide complementary to 18S rRNA as a control for loading. The graph represents densitometric analysis of the autoradiograph expressed relative to control at 3 h.  
30 B) MCF-7 cells proliferating in medium supplemented with 5% FCS were treated with ORG 2058 (10nM), ORG 2058 + RU 486 (100 nM)

simultaneously or ethanol vehicle and harvested from Northern analysis at 3 h. The Northern blot was probed with a 1.8 kb cDNA sub-clone of PRG1 and with an oligonucleotide complementary to 18S rRNA as a control for loading.

5 **Figure 8: Effect of the protein synthesis inhibitor cycloheximide on ORG 2058 induction of PRG1 mRNA.**

10 A) T-47D cells proliferating in insulin-supplemented serum-free medium were treated with ORG 2058 (10 nM), cycloheximide (CHX, 20 µg/ml), ORG 2058 and CHX simultaneously or ethanol vehicle and harvested for Northern analysis at 3 h. The Northern blot was probed with the PRG1 fragment of PRG1 and with an oligonucleotide complementary to 18S rRNA as a control for loading. The graph represents densitometric analysis of the autoradiograph expressed relative to control at 3 h.

15 B) T-47D cells proliferating in medium supplemented with 5% charcoal-treated FCS were treated with livial (10 nM) and ethanol vehicle in the presence and absence of actinomycin D (5 µg/ml) and harvested for Northern analysis. The Northern blot was probed with a 1.8 kb cDNA sub-clone of PRG1. C, ethanol control; L, livial.

20

**Figure 9: PRG1 mRNA regulation by breast cancer cell mitogens.**

Northern blots were probed with a 1.8 kb cDNA sub-clone of PRG1 and with an oligonucleotide complementary to 18S rRNA as a loading control.

25 Densitometric analysis, adjusted for loading, of the autoradiographs is presented.

30 A) Total RNA was isolated from T-47D cells growth arrested by serum deprivation and then stimulated to progress through the cell cycle by addition of insulin (1.7 µM), bFGF (55 pM) or heregulin (5 nM). PRG1 mRNA levels were measured at 3 or 4 h. and expressed relative to time matched vehicle treated controls.

35 B) Total RNA was isolated from MCF-7 cells rescued with estrogen (17β estradiol, 100 nM) following pre-treatment with antiestrogen (ICI 182780, 10 nM) for 48 h. in 5% fetal calf serum. PRG1 mRNA levels were measured at 4 h. and expressed relative to the average of antiestrogen treated control samples.

C) Total RNA was isolated from T-47D cells growth arrested by serum deprivation or exponentially growing in medium containing 10% fetal calf serum. PRG1 mRNA levels from exponentially growing cells are expressed relative to PRG1 mRNA levels from growth arrested cells.

5

**Figure 10: Affinity purification of FLAG PRG1 fusion protein.**

PRG1 fusion protein was expressed in bacteria, purified samples run on a 10% SDS-PAGE gel and Western blotted with an anti-FLAG antibody as described in the Materials and Methods. Lane 1, molecular weight markers; lane 2, soluble bacterial lysate as loaded on column; lanes 3-6, flow through; lanes 7-9, washes 1, 4, 5; lanes 10-15, fractions 1-6. The position of the FLAG PRG1 fusion protein is marked with an arrow.

15

**Example:**

**Materials and Methods**

**Reagents**

20 Steroids and growth factors were obtained from the following sources: ORG 2058 (16 $\alpha$ -ethyl-21-hydroxy-19-norpregn-4-en-3,20-dione), Amersham Australia; R5020 (17 $\alpha$ -21-dimethyl-19-norpregn-4,9-diene-3,20-dione), Du Pont (Australia) Ltd; MPA (17 $\alpha$ -acetoxy-6 $\alpha$ -methyl-4-pregn-4-en-3,20-dione), Dr. Dudley Jacobs of Upjohn Pty Ltd, Australia; livial ((7 $\alpha$ , 17 $\alpha$ )-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one), Dr. William Schoonen of Organon International, Oss, The Netherlands; RU 486 (17 $\beta$ -hydroxy-11 $\beta$ -(4-methylaminophenyl)-17 $\alpha$ -(1-propynyl)-estra-4,9-diene-3-one), Dr John-Pierre Raynaud of Roussel-Uclaf, Romainville, France; ICI 182780 (7 $\alpha$ -[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]estra-1,3,5(10)-triene-3,17 $\beta$ -diol) was a gift from Dr. Alan Wakeling, (Zeneca Pharmaceuticals, Macclesfield, UK); dexamethasone (9-fluoro-11,17,21-trihydroxy-16-methylpregn-1,4-diene-3,20-dione) and 17 $\beta$ -estradiol, Sigma Chemical Co., St. Louis, Mo.; human insulin, Actrapid; CSL-Novoo, Australia; human recombinant bFGF was donated by Dr. A. Protter, Pacific Biotechnology, Sydney, Australia and recombinant heregulin was produced by Drs. Rod Fiddes, CRC for Biopharmaceutical Research and Dr. Roger Daly, Garvan

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Institute of Medical Research (32). Steroids were stored at -20°C as 1000-fold-concentrated stock solutions in absolute ethanol. Growth factors were stored at -20°C and diluted on the day of use; bFGF in 30 µM human transferrin (Sigma Chemical Co., St. Louis, MO); heregulin as described (32). Insulin was stored at 4°C and used diluted directly in tissue culture medium. Cycloheximide (Calbiochem-Behring Corp., La Jolla, CA) was dissolved at 20 mg/ml in water and filter sterilized. Cosmegen, actinomycin D, (Merck Sharp and Dohme Research Pharmaceuticals, Rahway, NJ) was dissolved at 0.5 mg/ml in sterile water and used immediately. Tissue culture reagents were purchased from standard sources.

### Cell Culture

The sources and maintenance of the human breast cancer cell lines used in this study were as described previously (33). 184 and 184B5 normal breast epithelial cells were the kind gift of Dr. M. Stampfer (University of California, Berkley, CA) and were maintained in mammary epithelial growth medium (Clonetics, San Diego, CA). Tissue culture experiments in serum-free medium were performed as previously described (6, 27). Briefly, T-47D cells were taken from stock cultures and passaged for 6 days in phenol red-free RPMI medium supplemented with 10% charcoal-treated fetal calf serum (FCS). During this time the cells received two changes of medium at 1- to 3-day intervals. The cells were replated into replicate 150 cm<sup>2</sup> flasks in medium containing 10% charcoal-treated FCS and the medium replaced with serum-free medium on the next two days. Serum-free medium was supplemented with 300 nM human transferrin. In experiments involving ORG 2058 the final serum-free medium contained 10 µg/ml (1.7 µM) human insulin. Three days after completion of these pre-treatments growth factor, steroid, steroid antagonist or cycloheximide were added. Control flasks received vehicle to the same final concentration. Cell cycle phase distribution was determined by analytical DNA flow cytometry, as previously described (6, 34).

Tissue culture experiments in serum-containing medium were performed as previously described (34). The experiment with livial and actinomycin D was as for experiments in serum-containing medium except that the medium contained 5% charcoal-treated FCS. Actinomycin D was added at the same time as livial. The experiment involving "estrogen rescue"

was performed as described (30). Briefly, MCF-7 cells were cultured in medium containing 5% FCS for 2 days. The antiestrogen ICI 182780 (10 nM) was then added to the medium for 48 h. after which the growth-arrested cells were treated with 100 nM 17 $\beta$ -estradiol, also added directly to the medium, resulting in the synchronous re-entry of cells into the cell cycle.

#### **RNA isolation and Northern Analysis**

Cells harvested from triplicate 150 cm<sup>2</sup> flasks were pooled and RNA extracted by a guanidinium-isothiocyanate-caesium chloride procedure and Northern analysis was performed as previously described with 20  $\mu$ g of total RNA per lane (6, 11). The membranes were hybridized overnight at 50°C with probes labelled with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham Australia Pty Ltd, Castle Hill, Australia) using the Random Prime Labelling Kit (Promega, Sydney, Australia). The membranes were washed at a highest stringency of 0.2 x SSC (30 mM NaCl, 3 mM sodium citrate [pH 7.0]) + 1% sodium dodecyl sulfate at 65°C and exposed to Kodak X-OMAT or BIOMAX film at -70°C. Human multiple tissue Northern blots (Clontech Laboratories, Inc. Palo Alto, CA) were hybridized under conditions recommended by the manufacturer. The mRNA abundance was quantified by densitometric analysis of autoradiographs using Molecular Dynamics Densitometer and software (Molecular Dynamics, Sunnyvale, CA). The accuracy of loading was estimated by hybridizing membranes with a [ $\gamma$ -<sup>32</sup>P]ATP end-labelled oligonucleotide complementary to 18S rRNA.

#### **Differential Display**

Differential display was carried out as described (35). Total RNA, 200 ng, obtained from T-47D cells treated with the synthetic progestin ORG 2058 for 3 h. or from T-47D cells treated with ethanol control was reverse transcribed with 5'-T<sub>12</sub>GG as the primer. The cDNA products were amplified by the polymerase chain reaction (PCR) using 5'-T<sub>12</sub>GG and 5'-CAAACGTCGG primers. The PCR products were separated on a 6% polyacrylamide denaturing sequencing gel. The PCR product of interest was excised from the gel, re-amplified by PCR and cloned into the pGEM-T vector (Promega). DNA sequencing was performed by the dideoxy chain termination method using T7 DNA polymerase (AMRAD Pharmacia Biotech, Melbourne, Australia) and Sequenase 2.0 kit (Bresatec, Adelaide, Australia) or by cycle sequencing

using the *fmol*® DNA Cycle Sequencing System (Promega). Sequence database searches were performed at the NCBI using the BLAST network service.

5     **Library Screening**

          The differential display technique generates small (<500 nt) cDNA fragments. In order to obtain additional cDNA sequence lambda cDNA libraries derived from human kidney (Clontech,  $2.85 \times 10^5$  pfu) and human heart (Stratagene, La Jolla, CA  $6 \times 10^5$  pfu) were screened using the  $^{32}\text{P}$ -  
10     labelled differential display cDNA fragment as a probe under stringent hybridization conditions. Seven strongly hybridizing clones were isolated and excised for sequencing using bacterial strain XL1-Blue. Sequencing was performed as described above. Amino acid sequence alignments were performed using the computer programs MacVector™ 4.5.3 and SeqVu.

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**Expression of PRG1 protein in bacteria**

          Clones 11.2 and 19.1 (Figure 2) were cloned as Eco R1 fragments into pBluescript (pBS11.2 and pBS19.1). An Xba 1/Nco 1 fragment from pBS19.1 was then ligated to replace an Xba 1/Nco 1 fragment from pBS11.2.  
20     The resultant plasmid contained the complete PRG1 open reading frame. PCR using this plasmid and the forward primer 5'ATGAATTCATGCCGTTGGAAGTACGCAGAGC-3' and the reverse primer 5'-TACCTAGTCGACTCAGTGTTTCCTGGAGGAGTCAGC-3' was then performed to generate a full length open reading frame sequence with the  
25     appropriate terminal sequences that could next be cut with Eco R1 and Sal 1. The resulting fragment was cloned into the bacterial expression vector pFLAG=AE-2 (Eastman Kodak Company, New Haven, CT, USA). *E. coli* (DH5a strain) were then transformed with this construct and used to inoculate L-broth (containing 0.4% glucose) at a 1:100 dilution. The  
30     culture was grown to OD600 approx 0.4. Expression of the FLAG PRG1 fusion protein was induced by the addition of IPTG (0.5mM final concentration), continuing incubation for 1 hr at 30° C, shaking at 240rpm.

          To prepare the soluble fraction, bacteria from a 500ml culture were resuspended in 50 ml of extraction buffer A (50 mM Tris HCl, pH 8.0, 5mM EDTA, 0.25 mg/ml lysozyme, 50 µg/ml sodium azide). Extraction Buffer  
35

B (5 ml) was then added (1.5 M NaCl, 0.1 M CaCl<sub>2</sub>, 0.1 M MgCl<sub>2</sub>, 0.02 mg/ml DNase I, 0.05 mg/ml aprotonin). The bacterial lysate was then centrifuged at 25000g for 1 hour. The supernatant represents the soluble lysate fraction. To purify the PRG1 fusion protein, 50 ml of the soluble fraction was loaded  
5 onto a column containing 1 ml of anti-FLAG M2 affinity gel (Eastman Kodak Company, New Haven, CT, USA). The column flow rate was 0.5 ml/min. The column was then washed with 4 x 9ml of TBS (50mM Tris-HCl, 150mM NaCl at a final pH of 7.4). The bound fusion protein was then eluted with 0.1 M glycine pH 3.0 (1 ml + 10x 0.5 ml). Aliquots from each fraction were run on  
10 a 10 % SDS -PAGE gel together with pre-stained molecular weight markers (Sigma) and Western blotted with an anti-FLAG M2 antibody (Eastman Kodak Company, New Haven, CT, USA).

## Results

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### Cloning of a cDNA identified by differential display

The differential display technique was used to identify mRNAs expressed in T-47D human breast cancer cells whose levels of expression had altered in response to treatment with the synthetic progestin ORG 2058 for 3  
20 h. Using the PCR primer combination 5'-T<sub>12</sub>GG and 5'-CAAACGTCGG a total of 9 cDNA fragments identified by gel electrophoresis were clearly upregulated (Fig. 1A). Preliminary confirmatory screening by Northern analysis showed one of the cDNA fragments, designated PIG1 (described in the Applicant's Australian Provisional Patent Application No. PN6144), was  
25 induced rapidly in the presence of ORG 2058 (Fig. 1B) and therefore warranted further characterisation.

In order to obtain the complete coding sequence from which PIG1 was derived, a human kidney cDNA library constructed using oligo-dT-primed and random-primed cDNA was screened using the PIG1 fragment.  
30 Four cDNAs were isolated after screening 2.85 x 10<sup>5</sup> recombinants, namely 11.2, 6.3, 3.1 and 19.1 (Fig. 2A). Further screening of this library with an oligonucleotide derived from 5' sequence of 3.1 (5'-ACCGTCATCGTCATCGGTGGG-3') and with a 373 nt *EcoR1-BglII* restriction fragment derived from 3.1.1 resulted in the isolation of a chimeric clone 9.1  
35 and clone 8.1 (Fig. 2A). Screening of a human heart library with the 373 nt *EcoR1-BglII* restriction fragment resulted in the isolation of clone H7.1 (Fig.



2A). Clones 11.2, 6.3, 3.1 were sequenced in their entirety on both strands, and 350 nucleotides of the 5' end of clone 19.1 were sequenced on both strands, to give 2887 nt of cDNA sequence, designated PRG1, shown in uppercase lettering (Fig. 2B). While the 2887 nt of sequence is less than the mRNA size determined from Northern blots it contains a complete open reading frame as discussed below.

Comparison of the cDNA sequence with the GenBank and EMBL databases revealed a partial overlap with a cosmid clone (CRI-JC2015) (36) containing human genomic sequence from chromosome 10. Nucleotides numbering 1-399 of the cDNA overlap with nucleotides 1671-2070 from the cosmid clone but in the reverse orientation (Fig. 2A). Nucleotides 1670-1 of the cosmid clone are not present in PRG1 and appear to be intron sequence as the consensus splicing nucleotides, GT, which commonly flank the start of introns (37) are present at bases 1670 and 1669, respectively. Extrapolating backwards 27 nucleotides from the 5' end of the cDNA into the genomic sequence reveals an in-frame stop codon, refer to Fig. 2B where the genomic sequence is shown in lowercase lettering.

Analysis of the PRG1 cDNA sequence identified an open reading frame (ORF) containing 520 amino acids encoding a polypeptide very similar to human liver 6-phosphofructo-2-kinase/fructose--2,6-bisphosphatase (PFK-2/FBPase-2) (38) as well as to bovine brain and heart forms of this enzyme (39, 40). PRG1 bears 72% amino acid identity with the human liver PFK-2/FBPase-2 in 447 amino acid overlap and 93% and 74% identity with bovine brain PFK-2/FBPase-2 and bovine heart PFK-2/FBPase-2 in 462 amino acid and 447 amino acid overlap, respectively (Fig. 3). The ORF identified in the PRG1 cDNA sequence appears to be complete. The initiation codon preceded by an in-frame stop codon located 357 nucleotides upstream as identified in the cosmid clone (CRI-JC2015) and the initiating methionine is within close proximity of initiating methionines of other known related polypeptides (examples 38, 40-42) with the exception of the bovine brain PFK-2/FBPase-2, the sequence of which is incomplete (39).

Analysis of the deduced amino acid sequence of PRG1 using the Prosite Database Release 13.0 revealed several consensus phosphorylation sites, three for cyclic AMP and cyclic GMP dependent protein kinases beginning at residues 53, 189, 458, seven for protein kinase C beginning at residues 52, 129, 272, 441, 471, 516, 517, eleven for casein kinase II at

residues 129, 153, 171, 192, 333, 340, 362, 403, 441, 478, 512 as well as two for tyrosine kinases beginning at residues 348, 402. Consensus sequences for one N-glycosylation site, three N-myristoylation sites and two amidation sites were detected beginning at residues 128, 118, 266, 509, 218, 274,

5 respectively. In addition the ATP/GTP-binding site signature motif, conserved in all mammalian forms of PFK-2/FBPase-2 (43), was identified as amino acids 42-49 as was the phosphoglycerate mutase family phosphohistidine signature, amino acids 51-60.

#### 10 Northern blot analysis of PRG1 gene expression

The expression profile of PRG1 in a panel of human breast cancer and normal breast cell lines was investigated by hybridizing Northern blots of total RNA isolated from 1 normal breast epithelial cell strain (HMEC 184), 2 transformed normal epithelial cell lines (HMEC 184B5 and HBL-100) and 15 12 breast cancer cell lines (only 9 are shown) to a probe from a 1.8 kilobase (kb) cDNA sub-clone, 6.3.1 (Fig. 2A), which contains 98% of the PRG1 ORF. PRG1 mRNA, a single transcript of -4.4 kb, was expressed in all the breast cancer and normal breast epithelial cell lines examined (Fig. 4A). The highest level of expression was in the T-47D cell line and the lowest levels were noted in SK-BR-3 (not shown), BT-474, HBL-100, HMEC 184 and HMEC 20 184B5 cell lines. There was no correlation between PRG1 mRNA expression and estrogen receptor (ER), progesterone receptor (PR) or glucocorticoid receptor (GR) status (44) although the T-47D cell line, which expresses PR at a level 5-fold higher than the other cell lines had the highest level of expression (45).

The tissue specificity of PRG1 gene expression was also investigated by hybridizing Northern blots of poly A<sup>+</sup> RNA isolated from a variety of human tissues to a probe made from the sub-clone 6.3.1. A 4.4 kb transcript was detected in all the tissues examined (Fig. 4B). The apparent abundance of PRG1 mRNA in skeletal muscle shown in Fig. 4B may be the result of 30 uneven mRNA loading as in a second set of human tissue poly A<sup>+</sup> Northern blots skeletal muscle mRNA levels were similar to those of the kidney. Likewise, colon showed a higher level of expression in the second set of Northern blots. In the heart and skeletal muscle a band of 1.35 kb was also 35 detected. This band was more easily detected under lower stringency conditions when it was also found to be present in kidney, pancreas, skeletal

muscle and colon (data not shown) and suggests that the cDNA PRG1 probe was cross-reacting with a related sequence. A third transcript of around 9.5 kb was also detected in skeletal muscle.

5     **PRG1 mRNA is induced transiently by progestin**

To examine in detail the kinetics of progestin induction of PRG1, regulation of PRG1 mRNA expression was investigated in T-47D cells cultured in insulin-supplemented serum-free medium and harvested for mRNA at various time points following ORG 2058 treatment (Fig. 5). PRG1  
10 detected a single mRNA species of approximately 4.4 kb in cells cultured in the absence of ORG 2058. The induction of PRG1 mRNA by ORG 2058 was an early and transient event. Maximal levels of PRG1 induction, 4.4-fold relative to time-matched control in the experiment shown in Fig. 5, were observed at 3 h. following treatment. The induction at 3 h. was typically  
15 between 2 and 4.4-fold relative to time-matched controls. After 6 h. mRNA levels had decreased and by 12 h. had returned to control levels. A more detailed analysis of early time-points showed that maximal levels were reached by 2 h. and sustained until 4 h. (data not shown). The increase in PRG1 preceded increases in the proportion of cells in S phase (data not  
20 shown), which typically occur around 10 h. in this system (6).

**Induction of PRG1 mRNA in breast cancer cell lines is mediated via the progesterone receptor**

To determine whether the effects of ORG 2058 on PRG1 expression  
25 were likely to be mediated by the PR we examined the effects of other synthetic progestins and the antiprogestin RU 486 in a variety of breast cancer cell lines. T-47D cells, growing exponentially in medium containing 5% FCS, were treated in parallel with the synthetic ORG 2058, R5020 and MPA at 10 nM and harvested for mRNA at 3 h. All 3 synthetic progestins  
30 induced PRG1 mRNA between 2- and 2.5-fold above control levels (data not shown). PRG1 mRNA was also induced by the synthetic progestin livial (10 nM) in T-47D cells growing in the presence of 5% charcoal-treated FCS as discussed later. The effect of ORG 2058 on PRG1 mRNA in another PR-positive cell line (MCF-7) and in a PR-negative cell line (MDA MB-231) were  
35 investigated. ORG 2058 increased PRG1 mRNA in MCF-7 cells approximately 2-fold above control mRNA levels at 3 h. (Fig. 6A). In contrast

in MDA-MB-231 cells the levels of PRG1 mRNA in the presence of ORG 2058 were decreased approximately 30% below control mRNA levels at 3 h. with recovery at 6 h. (Fig. 6B).

Additional evidence for the involvement of PR in mediating progestin effects on PRG1 was obtained using the progestin antagonist RU 486. This compound acts as a competitive inhibitor of the binding of progestins to the PR (46) and its effect on the induction of PRG1 mRNA was investigated by treatment of T-47D cells with ORG 2058 (10 nM) and RU 486 (100 nM) either alone or simultaneously in serum-free medium supplemented with insulin. The cells were harvested 3 h. after the treatment, when PRG1 mRNA levels were at a maximum. Simultaneous administration of ORG 2058 and RU 486 led to complete inhibition of progestin-induced PRG1 expression, while treatment with RU 486 alone had no effect on mRNA levels (Fig. 7A). Similar effects were seen in MCF-7 cells grown in the presence of serum although the abrogating effect of RU 486 was not quite as pronounced (Fig. 7B).

Given that the consensus sequence for the glucocorticoid and progesterone response elements is similar (47) one might expect that glucocorticoids could regulate PRG1 expression via the glucocorticoid receptor (GR). To investigate this possibility to GR-positive cell lines, MCF-7 and MDA MB-231, were treated with the synthetic glucocorticoid dexamethasone (100 nM) and harvested at 3 and 6 h. following treatment. No increase in PRG1 mRNA was observed at these times (Fig. 6A and 6B). Similarly, no increase in PRG1 mRNA was observed in T-47D cells, which also express GR, following treatment with dexamethasone for 3 h. (results not shown). In MDA-MB-231 cells, however, dexamethasone reduced PRG1 mRNA to approximately 60% of control levels at 3 h. with some recovery evident at 6 h. These data are consistent with the progestin effect being mediated via the PR.

#### **PRG1 induction by progestin does not require *de novo* protein synthesis**

To distinguish between direct activation of PRG1 transcription by the PR or indirect activation via the synthesis of intermediary proteins, T-47D cells were treated with ORG 2058 (10 nM) in the presence of the protein synthesis inhibitor, cycloheximide (20 µg/ml). Cycloheximide failed to block the progestin-mediated induction of PRG1 mRNA. Treatment with

cycloheximide alone resulted in an increase of PRG1 mRNA to a level similar to that achieved by ORG 2058 alone, while in the presence of both ORG 2058 and cycloheximide there was "superinduction" of PRG1 mRNA (Fig. 8A). The magnitude of this induction, 12-fold in the experiment shown in Fig. 8, was larger than expected from a combination of the responses to the individual compounds. Such superinduction involving protein synthesis inhibitors is characteristic of genes such as  $\beta$ - and  $\gamma$ -actin, *c-fos* and *c-myc* which are switched on early (0-2 h.) following stimulation of cells by mitogens (48-50). Cycloheximide has stabilising effects on mRNA (51), prolongs transcription (48) and can itself act as a nuclear signalling agonist (52) all of which can contribute to the superinduction effect. PRG1 mRNA was not induced in T-47D cells treated with the synthetic progestin livial (10 nM) in the presence of the transcription inhibitor actinomycin D (5  $\mu$ g/ml) (Fig. 8B). Together these data suggest that the induction of PRG1 mRNA is due to the direct action of the PR on PRG1 transcription and does not require *de novo* protein synthesis.

#### **Regulation of PRG1 expression by breast cancer cell mitogens**

Given that expression of rat F-type PFK-2/FBPase-2 is linked to the proliferation state of cells (53) and PRG1 mRNA levels increase prior to the progestin-induced increases in S phase cells, the effect of other known breast cancer cell mitogens on PRG1 expression was examined. T-47D cells were growth-arrested in G<sub>1</sub> phase by serum deprivation and stimulated to re-initiate cell cycle progression with insulin, heregulin or basic fibroblast growth factor. These mitogens stimulate cell cycle progression with increases in the S phase population first evident around 12-15 h. and maximal at about 24 h. (32, 54). The induction of PRG1 mRNA due to insulin, heregulin and bFGF were 2.8-, 2.6- and 1.9-fold, respectively at 3 or 4 h. (Fig. 9A). Heregulin has previously been reported to be one of the most potent mitogens for T-47D cells (32) whereas insulin and bFGF are equipotent (54) and therefore the degree of induction of PRG1 mRNA was unrelated to the potency of the mitogens in stimulating cell cycle progression. The regulation of PRG1 mRNA expression by estrogen was examined in MCF-7 breast cancer cells using a model in which cells cultured in serum were growth-arrested in the G<sub>1</sub> phase of the cycle by the antiestrogen ICI 182780 and then stimulated to re-enter the cell cycle with

17 $\beta$ -estradiol (30). In this system increases in S phase begin around 12 h. and are maximal between 21 and 24 h. Induction of PRG1 mRNA (2.0-fold) was observed at 4 h. (Fig. 9B). A comparison was also made between T-47D cells growth-arrested by serum deprivation and cells exponentially growing in medium supplemented with 10% fetal calf serum. PRG1 mRNA was detected at very low levels in growth-arrested T-47D cells in unsupplemented serum-free medium while cells cultured in the presence of serum expressed PRG1 mRNA at a 10.9-fold higher level (Fig. 9C). Therefore induction of PRG1 mRNA is not restricted to progestins, but is a common response to mitogenic stimulation. (Also refer to Table 1).

**Table 1: PRG1 mRNA regulation by breast cancer cell mitogens**

Cell type and treatment	Relative mRNA Expression
T-47D	
Exponentially growing cells <sup>‡</sup>	10.9 <sup>†</sup>
Insulin (1.7 $\mu$ M)	2.8 <sup>†</sup> at 4 h
Heregulin (5nM)	1.9 <sup>†</sup> at 4 h
bFGF (55 pM)	2.6 <sup>†</sup> at 3 h
MCF-7	
17 $\beta$ estradiol (100 nM)	2.0 $\pm$ at 4 h

- <sup>‡</sup> Cells were growing exponentially in medium containing 10% fetal calf serum.  
<sup>†</sup> T-47D cells were treated as described in Materials and Methods. PRG1 mRNA levels are expressed relative to levels in cells maintained in serum-free medium.  
 $\pm$  MCF-7 cells were rescued with 17 $\beta$  estradiol following pre-treatment with the antiestrogen ICI 182 780 for 48 h as described in Materials and Methods. PRG1 mRNA is expressed relative to antiestrogen treated control levels.

**Production of bacterially expressed PRG1 as a FLAG fusion protein.**

PRG1 was produced as a soluble protein in bacteria in the form of a FLAG fusion protein as described in the Materials and Methods. Figure 10 shows the results of affinity purification on an anti-FLAG M2 affinity column. The presence of the fusion protein has been detected by SDS-PAGE

of fractions and Western blotting with an anti-FLAG antibody. Unpurified bacterial lysate (soluble fraction) (ie the material loaded onto the affinity purification column) is run in lane 2 and a large band at the predicted molecular weight of 60kDa is present, with several minor lower molecular weight species present that are also antibody-reactive. Following washing (lanes 7-9), glycine was used to elute the fusion protein which was present in fractions 1-6 (lanes 10-15) at the expected molecular weight. Coomassie staining of this gel (not shown) suggests the majority of the fusion protein eluted in lanes 12 and 13 in a substantially pure form.

### Summary and conclusions

The progestin regulation of PRG1 mRNA was studied in some detail and Northern analysis of PRG1 mRNA levels over a 24 h. time period showed a rapid and transient induction by ORG 2058 that peaked at 3 h. and returned to control levels by 12 h. Several lines of evidence are consistent with the view that this response is mediated by the PR. First, the induction of PRG1 mRNA occurred in the presence of three other synthetic progestins, R5020, MPA and livial. Second, although these progestins potentially have some cross-reactivity with the GR (55, 56) the progestin induction does not appear to be mediated by this receptor and the glucocorticoid response element (GRE) as the glucocorticoid dexamethasone had no effect on PRG1 mRNA in T-47D or MCF-7 cell lines. Third, the progestin antagonist, RU 486, inhibited the progestin-induction of PRG1 mRNA. Interestingly, in cells expressing high levels of GR, i.e. MDA-MB-231 cells (44), dexamethasone and ORG 2058 caused a small but significant decrease in PRG1 mRNA levels. The mechanism by which this occurs is not known at this stage but may involve cross-reactivity of ORG 2058 with the GR which in this cell line is able to weakly down-regulate PRG1 mRNA expression.

The progestin induction of PRG1 mRNA is not prevented by the presence of the protein synthesis inhibitor cycloheximide but is blocked by the transcription inhibitor actinomycin D. This strongly suggests that induction of PRG1 by progestin is a direct transcriptional effect of ligand-activated PR on a putative PRE located in the PRG1 gene. A computer search of DNA sequence from the cosmid clone CRI-JC2015 encompassing 3000bp from the initiating methionine in a 5' direction for an optimal PRE sequence as determined by *in vitro* studies (57) revealed several PRE-like sequences.

Sequencing of PRG1 shows that PRG1 exhibits significant homology to genes in various species including human that encode the key regulatory bifunctional enzyme phosphofructokinase 2/fructose-2,6-biphosphatase (PFK-2/FBPase-2, EC.2.7.1.105/3.1.3.46) suggesting PRG1 may represent a related enzyme (see Figure 4). PFK-2/FBPase-2 controls levels of the compound fructose-2,6-bisphosphate (Fru-2,6-P<sub>2</sub>), which has a major role in the regulation of enzymes controlling glycolysis, gluconeogenesis, and lipogenesis. PRG1 may also control levels of Fru-2,6-P<sub>2</sub> or other as yet unidentified regulatory factors.

- PRG1 therefore appears to represent a novel gene with potential to be:
- (i) one of the few known genes to be directly regulated by progestins and hence an important mediator of progestin action and a marker of clinical responsiveness to progestins; and
  - (ii) a gene involved in cell cycle regulation by progestins and other mitogens and hence a new target for antiproliferative agents.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.



## References

1. Clarke CL, Sutherland RL 1990 Progesterin regulation of cellular proliferation 11:266-301
2. Shi YE, Liu YE, Lippman ME, Dickson RB 1994 Progestins and antiprogesterins in mammary tumour growth and metastasis Human Reproduction 9 supplement 1:162-173
3. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery Jr. CA, Shyamala G, Conneely OM, O'Malley BW 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities Gene Dev 9:2266-2278
4. Anderson TJ, Battersby S, King RJB, McPherson K, Going JJ 1989 Oral contraceptive use influences resting breast proliferation Hum Pathol 20: 1139-1144
5. Sedlacek SM, Horwitz KB 1984 The role of progestins and progesterone receptors in the treatment of breast cancer Steroids 44:467-84
6. Musgrove EA, Lee CSL, Sutherland RL 1991 Progestins both stimulate and inhibit breast cancer cell cycle progression while increasing expression of transforming growth factor  $\alpha$ , epidermal growth factor receptor, *c-fos*, and *c-myc* genes Mol Cell Biol 11:5032-5043
7. Alkhalaf M, Murphy LC 1992 Regulation of *c-jun* and *jun-B* by progestins T-47D human breast cancer cells Mol Endo 6:1625-1633
8. Murphy LC, Alkhalaf M, Dotzlaw H, Coutts A, Haddad-Alkhalaf B 1994 Regulation of gene expression in T-47D human breast cancer cells by progestins and antiprogesterins Human Reproduction 9 supplement 1:174-180

9. Joyeux C, Rochefort H, Chabos D 1989 Progesterone increases gene transcription and messenger ribonucleic acid stability of fatty acid synthetase in breast cancer cell Mol Endocrinol 4:681-686
- 5 10. Misrahi M, Loosfelt H, Atger M, Merial C, Zerah V, Dessen P, Milgrom E 1988 Organisation of the entire rabbit progesterone receptor mRNA and of the promoter and 5' flanking region of the gene Nucleic Acid Res 16:5459-5472
- 10 11. Alexandra IE, Clarke CL, Shine J, Sutherland RL 1989 Progesterone inhibition of progesterone receptor gene expression in human breast cancer cells Mol Endocrinol 3:1377-1386
- 15 12. Jantzen K, Fritton HP, Igo-Kemenes T, Espel E, Janich S, Cato AC, Mugele K, Beato M 1987 Partial overlapping of binding sequences for steroid hormone receptors and DNaseI hypersensitive sites in the rabbit uteroglobin gene regions Nucleic Acids Res 11:4535-4552
- 20 13. Theveny B, Bailly A, Rauch C, Rauch M, Delain E, Milgrom E 1987 Association of DNA bound progesterone receptors Nature 329:79-81
- 25 14. Di Lorenzo D, Albetini A, Zava D 1991 Progesterone regulation of alkaline phosphatase in the human breast cancer cell line T47D Cancer Res 51:4470-4475
- 30 15. Hagley RD, Hissom JR, Moore MR 1987 Progesterone stimulation of lactate dehydrogenase in the human breast cancer line T-47D Biochim Biophys Acta 930:167-172
- 35 16. Alexandra IE, Shine J, Sutherland RL 1990 Progesterone regulation of estrogen receptor messenger RNA in human breast cancer cells Mol Endocrinol 4:821-828
17. Roman SD, Clarke CL, Hall RE, Alexander IE, Sutherland RL 1992 Expression and regulation of retinoic acid receptors in human breast cancer cells Cancer Res 52:2236-2242

18. Murphy LC, Murphy LJ, Shiu RP 1988 Progesterone regulation of EGF-receptor mRNA accumulation in T-47D human breast cancer cells  
Biochem Biophys Res Commun 150: 192-6
- 5
19. Ormandy CJ, Graham J, Kelly PA, Clarke CL, Sutherland RL 1992 The effect of progesterone on prolactin receptor gene transcription in human breast cancer cells DNA Cell Biol 11:721-726
- 10
20. Papa V, Hartmann KK, Rosenthal SM, Maddux BA, Siiteri PK, Goldfine ID 1991 Progesterone induces down-regulation of insulin-like growth factor-I (IGF-I) receptors in human breast cancer cells: potential autocrine role of IGF-II Mol Endocrinol 5:709-717
- 15
21. Papa V, Reese CC, Brunetti A, Vigneri R, Siiteri PK, Goldfine ID 1990 Progesterone increases insulin receptor content and insulin stimulation of growth in human breast carcinoma cells Cancer Res 50:7858-7862
- 20
22. Murphy LC, Murphy LJ, Dubik D, Bell GI, Shiu, RP 1988 Epidermal growth factor gene expression in human breast cancer cells: regulation of expression by progesterone Cancer Res 48:4555-4560
- 25
23. Murphy LC, Dotzlaw H 1989 Regulation of transforming growth factor alpha and transforming growth factor beta messenger ribonucleic acid abundance in T-47D, human breast cancer cells Mol Endocrinol 3: 611-7
- 30
24. Gong Y, Anzai Y, Murphy LC, Ballejp G, Holinka CF, Gurpide E, Murphy LJ 1991 Transforming growth factor gene expression in human endometrial adenocarcinoma cells: regulation by progesterone Cancer Res 51:5476-81
- 35
25. Poutanen M, Isomaa V, Kainulainen K, Vihko R 1990 Progesterone induction of 17 beta-hydroxysteroid dehydrogenase enzyme protein in the T-47D human breast-cancer cell line Int J Cancer 46:897-901

26. Coutts A, Murphy LJ, Murphy LC 1994 Expression of insulin-like growth factor binding proteins by T-47D human breast cancer cells: regulation by progestins and antiestrogens *Breast Cancer Res Treat* 32:153-164
- 5
27. Musgrove EA, Hamilton JA, Lee CSL, Sweeney KJE, Watts CKW, Sutherland RL 1993 Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression *Mol Cell Biol* 13:3577-3587
- 10
28. Musgrove EA, Lee CSL, Buckley MF, Sutherland RL 1994 Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle *Proc Natl Acad Sci USA* 91:8022-8026
- 15
29. Musgrove EA, Sarcevic B, Sutherland RL 1996 Inducible expression of cyclin D1 in T-47D human breast cancer cells is sufficient for Cdk2 activation and pRB hyperphosphorylation *J Cell Biochem* 60:363-378
- 20
30. Prall OWJ, Sarcevic B, Musgrove EA, Watts CKW, Sutherland RL 1996 Estrogen regulation of cell cycle progression and cyclin/CDK function in human breast cancer cells submitted
- 25
31. Liang P, Pardee AB 1992 Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction *Science* 257:967-971
- 30
32. Fiddes RJ, Janes PW, Sanderson GM, Sivertsen SP, Sutherland RL, Daly RJ 1995 Heregulin (HRG)-induced mitogenic signalling and cytotoxic activity of a HRG/PE40 ligand toxin in human breast cancer cells *Cell Growth Differ* 6:1567-1577
- 35
33. Buckley MF, Sweeney KJE, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CK, Musgrove EA, Sutherland RL 1993 Expression and amplification of cyclin genes in human breast cancer *Oncogene* 8:2127-33

34. Musgrove EA, Wakeling AE, Sutherland RL 1989 Points of action of estrogen antagonists and a calmodulin antagonist within the MCF-7 human breast cancer cell cycle *Cancer Res* 49:2398-2404
- 5 35. Liang P, Averboukh L, Keyomarsi K, Sager R, Pardee AB 1992 Differential display and cloning of messenger RNAs from human breast cancer versus mammary epithelial cells *Cancer Res* 52:6966-6968
- 10 36. Zheng C.J, Ma NS-F, Dorman TE, Wang M-T, Braunschweiger K, Soares L, Schuster MK, Rothchild CB, Bowden DW, Torrey D, Keith TP, Moir DT, Mao J-I 1994 Development of 124 sequence-tagged sites and cytogenetic localisation of 217 cosmids for human chromosome 10 *Genomics* 22:55-67
- 15 37. Stryer L 1988 Chapter 5 Flow of Genetic Information, ed. W.H. Freeman and Company, New York, p 110-112
- 20 38. Lange AJ, Pilgis SJ 1990 Sequence of human liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase *Nucleic Acids Res* 18:3652
- 25 39. Ventura F, Ambrosio S, Bartrons R, El-Maghrabi MR, Lange AJ, Pilgis SJ 1995 Cloning and expression of a catalytic core bovine brain 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase *Biochem Biophys Res Commun* 209:1140-1148
- 30 40. Sakata J, Uyeda K 1990 Bovine heart fructose 6-phosphate, 2-kinase/fructose 2,6-bisphosphatase: complete amino acid sequence and localisation of phosphorylation sites *Proc Natl Acad Sci USA* 87:4951-4955
- 35 41. Lively MO, El-Maghrabi MR, Pilgis J, D'Angelo G, Colosia AD, Ciavola JA, Fraser BA, Pilgis SJ 1988 Complete amino acid sequence of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase *J Biol Chem* 263:839-849

42. Sakata J, Abe Y, Uyeda K 1991 Molecular cloning of the DNA and expression and characterisation of rat testes fructose-6-phosphate, 2-kinase: fructose-2,6-bisphosphatase J Biol Chem 266:15764-15770
- 5 43. Pilksi SJ, Claus TH, Kurland IJ, Langer AJ 1995 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signalling enzyme Annu Rev Biochem 64:799-835
- 10 44. Hall RE, Lee CSL, Alexandra IE, Shine J, Clarke CL, Sutherland RS 1990 Steroid hormone receptor gene expression in human breast cancer cells: inverse relationship between oestrogen and glucocorticoid receptor messenger RNA levels Int J Cancer 46:1081-1087
- 15 45. Sutherland RL, Hall RE, Pang GYN, Musgrove EA, Clarker CL 1988 Effect of medroxyprogesterone acetate in proliferation and cell cycle kinetics of human mammary carcinoma cells Cancer Res 48:5084-5091
- 20 46. Bardon S, Vignon F, Chalbos D, Rochefort H 1985 RU486, A progestin and glucocorticoid antagonist, inhibits the growth of breast cancer cells via the progesterone receptor J Clin Endocrinol Metab 60:692-697
- 25 47. Carson-Jurica MA, Schrader WT, O'Malley BW 1990 Steroid receptor family: structure and functions Endocr Rev 11:201-220
48. Elder PK, Schmidt LJ, Ono T, Getz MJ 1984 Specific stimulation of actin gene transcription by epidermal growth factor and cycloheximide Proc Natl Acad Sci USA 81:7476-7480
- 30 49. Greenberg ME, Ziff EB 1984 Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene Nature-311:433-438
- 35 50. Greenberg ME, Hermanowski AL, Ziff EB 1986 Effect of protein synthesis inhibitors on growth factor activation of *c-fos*, *c-mys*, and actin gene transcription Mol Cell Biol 6:1050-1057

51. Rahmsdorf HJ, Schönthal A, Angel P, Litfin M, Rüther U, Herrlich P 1987 Posttranscriptional regulation of *c-fos* mRNA expression Nucleic Acids Res 15:1643-1659
- 5 52. Edwards DR, Mahadevan LC 1992 Protein synthesis inhibitors differentially superinduce *c-fos* and *c-jun* by three distinct mechanisms: lack of evidence for labile repressors EMBO J 11:2415-2424
- 10 53. Darville MI, Antoine IV, Mertens-Strijthagen JR, Dupriez VJ, Rousseau GG 1985 An E2F-dependent late-serum-response promoter in a gene that controls glycolysis Oncogene 11:1509-1517
- 15 54. Musgrove EA, Sutherland RL 1993 Acute effects of growth factors on T-47D breast cancer cell cycle progression European Journal of Cancer 29A:2273-2279
- 20 55. Poulin R, Baker D, Poirier D, Labrie F 1989 Androgen and glucocorticoid receptor-mediated inhibition of cell proliferation by medroxyprogesterone acetate in ZR-75-1 human breast cancer cells Breast Cancer Res Treat 13:161-172
- 25 56. Poulin R, Baker D, Poirier D, Labrie F 1990 Multiple actions of synthetic 'progestins' on the growth of ZR-75-1 human breast cancer cells: An *in vitro* model for the simulations assay of androgen, progestin, estrogen and glucocorticoid agonistic and antagonistic activities of steroids Breast Cancer Res Treat 17:197-210
- 30 57. Lieberman B.A., Bona B.J., Edwards D.P., Nordeen S.K., 1993, The Constitution of a progesterone response element, Molecular Endocrinology 7: 515-527.
- 35 58. Van Schaftingen, E. et al. 1982, European Journal of Biochemistry 129: 191-195

59. El-Maghrabi, M.R. et al. 1982, Journal of Biological Chemistry 257: 7603-7607.



CLAIMS:

1. An isolated DNA molecule comprising a nucleotide sequence more than 80% homologous to any one of the nucleotide sequences shown at;
  - 5 (i) Figure 2B from nucleotide 1 to 3018,
  - (ii) Figure 2B from nucleotide 1 to 470,
  - (iii) Figure 2B from nucleotide 141 to 3018, and
  - (iv) Figure 2B from nucleotide 470 to 2103.
- 10 2. An isolated DNA molecule comprising a nucleotide sequence more than 90% homologous to any one of the nucleotide sequences shown at;
  - (i) Figure 2B from nucleotide 1 to 3018,
  - (ii) Figure 2B from nucleotide 1 to 470,
  - (iii) Figure 2B from nucleotide 141 to 3018, and
  - 15 (iv) Figure 2B from nucleotide 470 to 2103.
- 20 3. An isolated DNA molecule comprising a nucleotide sequence substantially corresponding to, or complementary to, the nucleotide sequence shown at Figure 2B from nucleotide 1 to 3018.
4. An isolated DNA molecule comprising a nucleotide sequence substantially corresponding to, or complementary to, the nucleotide sequence shown at Figure 2B from nucleotide 1 to 470.
- 25 5. An isolated DNA molecule comprising a nucleotide sequence substantially corresponding to, or complementary to, the nucleotide sequence shown at Figure 2B from nucleotide 141 to 3018.
- 30 6. An isolated DNA molecule comprising a nucleotide sequence substantially corresponding to, or complementary to, the nucleotide sequence shown at Figure 2B from nucleotide 470 to 2103.
7. A DNA molecule according to any one of the preceding claims, said DNA molecule being of human origin.

8. A DNA molecule according to claim 7, said DNA molecule being of human kidney cell or breast cancer cell origin and/or encodes a protein normally expressed in human kidney cells, breast tissue or tumour cells.
- 5 9. An expression vector comprising a DNA molecule according to any one of the preceding claims.
- 10 10. A recombinant host cell including a DNA molecule according to any one of claims 1 to 8 or an expression vector according to claim 9.
11. A polynucleotide molecule capable of being utilised as a specific probe or primer for a DNA molecule according to any one of claims 1 to 8.
- 15 12. A polynucleotide molecule according to claim 7 being of a length greater than 10 nucleotides.
- 20 13. A polypeptide in a substantially pure form, said polypeptide comprising an amino acid sequence substantially corresponding to that shown at Figure 2B or an enzymatic portion thereof.
14. A polypeptide in a substantially pure form, said polypeptide consisting of an amino acid sequence substantially corresponding to that shown at Figure 2B.
- 25 15. An antibody which specifically binds to a polypeptide according to claim 13 or 14.
16. An assay for assessing progestin-responsiveness in a subject comprising the steps of;
- 30 (i) isolating cells or tissue from said subject; and
- (ii) detecting the presence of a polypeptide comprising an amino acid sequence substantially corresponding to that shown at Figure 2B.
- 35 17. An assay according to claim 16, wherein said step of detecting involves treating the isolated cells or tissue with an antibody according to

claim 15 and determining the presence of antibody bound to said polypeptide.

18. An assay according to claim 16, wherein said step of detecting  
5 involves treating the isolated cells or tissue with a substrate for said polypeptide, said substrate being normally converted by said polypeptide to a readily detected product.

19. An isolated DNA molecule comprising a progestin-regulatory  
10 element(s) (PRE) derived from a DNA molecule according to any one of claims 1 to 8.

20. A method for identifying agonist or antagonist compounds of the PRG1 polypeptide comprising the steps of;  
15 (i) subjecting an *in vitro* system containing PRG1 polypeptide, or an enzymatic portion thereof, to a test compound, and  
(ii) detecting any modulation of enzyme activity of said PRG1 polypeptide or enzymatic portion thereof.

21. A method according to claim 20, wherein step (ii) comprises  
20 detecting any modulation in 6-phosphofructo-2-kinase activity of said PRG1 polypeptide.

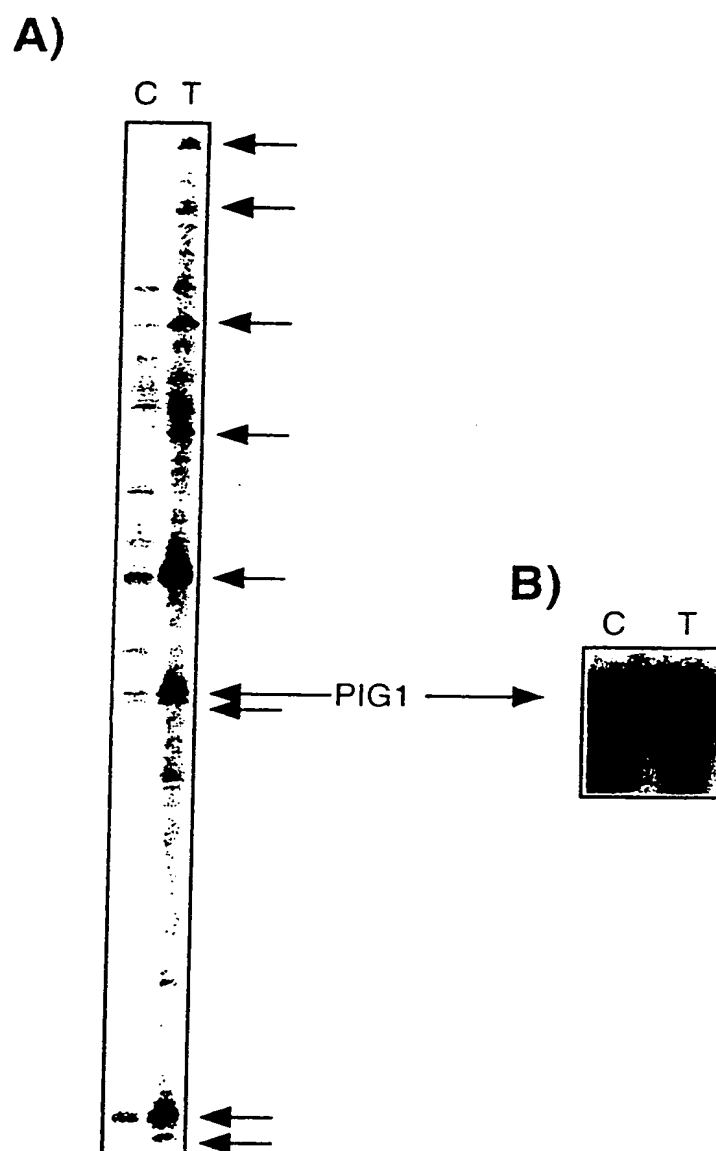
22. A method according to claim 20, wherein step (ii) comprises  
25 detecting any modulation in fructose-2,6-biphosphatase activity of said PRG1 polypeptide.

23. A method according to any one of claims 20 to 22, wherein said *in vitro* system comprises a whole cell lysate of cells expressing PRG1.  
30

24. An agonist or antagonist compound identified by the method of  
any one of claims 20 to 23.

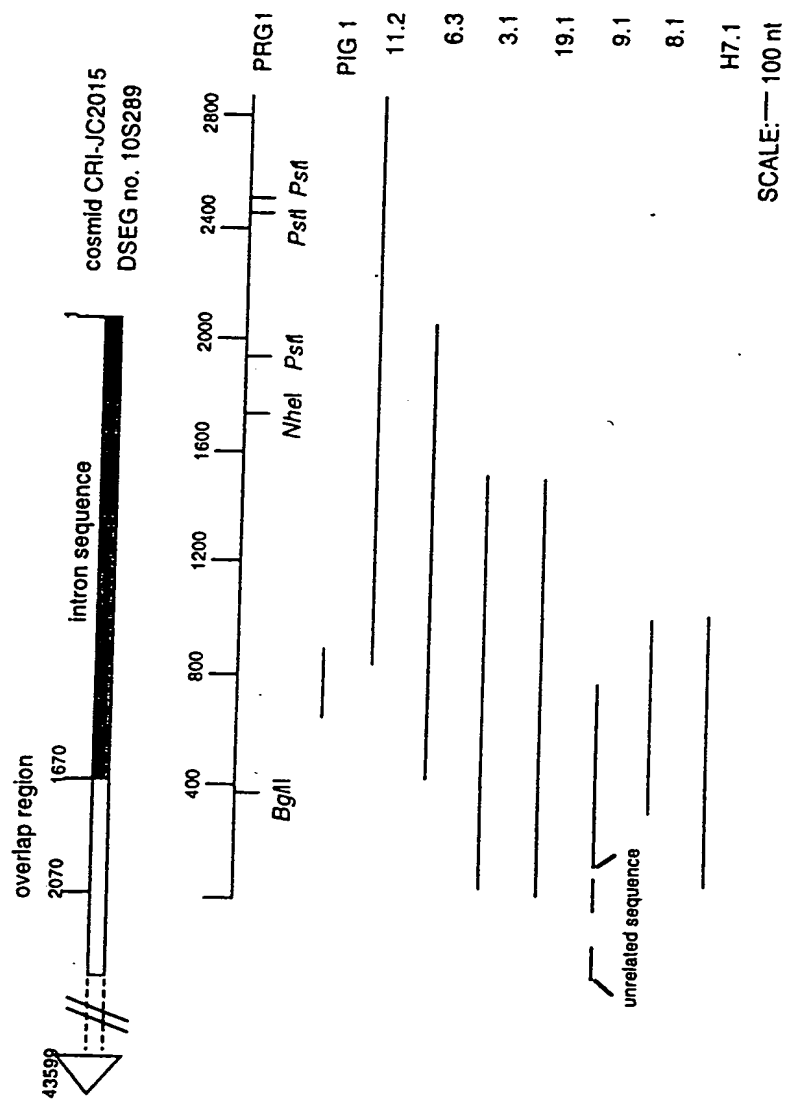
1/13

FIGURE 1



2/13

FIGURE 2A



3/13

caggctgcttccccggctcgccccaccctcctccccacgtggaagggggctggg 52  
 acccaaggaatgcggccccgccccgaggtgacgtacgcgtctgcggccagcc  
 cggactctttaaagccggcggtgcgcggggcatccCAGCCAAGCCGGAGAG 156  
 GAGGCGAGCGGCAGGGCCTGGTGGCGAGAGCGCGGCTGTCACTGCGCCCCGAG  
 CATCCCAGAGCTTTCCGAGCGGACGAGCCGGCCGTGCCGGGCATCCCCAGCC 260  
 TCGCTACCCCTCGCAGCACACGTTCGAGCCCCGCACAGGCGAGGGTCCGGAAC  
 TAGCCCCAAAGCACGTTTCCCCTGGCAGCGCAGGAAACGCCCGGCCGCGCGCC 364  
 GGCGCACGCCCCCTCTCCTCCTTTGTTCGGGGGTTCGGCGCCGCTCTCCT  
 GCCAGCGTCGGGATCTCGGCCCCGGGAGGCGGGCCGTTCGGGCGCAGCCGCGA 468  
 AGATGCCCGTTGGAAC TGACGCAGAGCCGAGTGCAGAAGATCTGGGTGCCCGT  
 M P L E L T Q S R V Q  
 GGACCACAGGCCCTCGTTGCCCAGATCCTTGCGGCCAAAGCTGACCAACTCC 572  
 K I W V P V D H R P S L P R S C G  
 CCCACCGTCATCGTCATGGTGGGCCTCCCCGCCCGGGGCAAGACCTACATCT  
 P K L T N S P T V I V M V G L P A  
 CCAAGAAGCTGACTCGCTACCTCAACTGGATTGGCGTCCCCACAAAAGTGAC 676  
 R G K T Y I S K K L T R Y L N W I G  
 TGTC AACGTGGGGAGTATCGCCGGGAGGCTGTGAAGCAGTACAGCTCCTACA  
 V P T K V F N V G E Y R R E A V K  
 TTCTTCCGCCCCGACAATGAGGAAGCCATGAAAGTCCGGAAGCAATGTGCCT 780  
 Q Y S S Y N F F R P D N E E A M K  
 TAGCTGCCTTGAGAGATGTCAAAGCTACCTGGCGAAAGAAGGGGGACAAAT  
 V R K Q C A L A A L R D V K S Y L A  
 TGCGGTTTTCGATGCCACCAATACTACTAGAGAGAGGAGACACATGATCCTT 884  
 K E G G Q I A V F D A T N T T R E  
 CATTTTGCCAAAGAAAATGACTTTAAGGCGTTTTCATCGAGTCGGTGTGCG  
 R R H M I L H F A K E N D F K A F  
 ACGACCCTACAGTTGTGGCCTCCAATATCATGGAAGTTAAATCTCCAGCCC  
 F I E S V C D D P T V V A S N I M E  
 GGATTACAAAGACTGCAACTCGGCAGAAGCATGGACGACTTCATGAAGAGG 988  
 V K I S S P D Y K D C N S A E A M  
 ATCAGTTGCTATGAAGCCAGCTACCAGCCCCCTCGACCCCGACAAATGCGACA  
 D D F M K R I S C Y E A S Y Q P L  
 GGGACTTGTCGCTGATCAAGGTGATTGACGTGGGCCGGAGGTTCTCTGGTGAA 1092  
 D P D K C D R D L S L I K V I D V G  
 CCGGGTGCAGGACCACATCCAGAGCCGCATCGTGTACTACCTGATGAACATC  
 R R F L V N R V Q D H I Q S R I V  
 CACGTGCAGCCGCGTACCATCTACCTGTGCCGGCACGGCGAGAACGAGCACA 1196  
 Y Y L M N I H V Q P R T I Y L C R  
 ACCTCCAGGGCCGCATCGGGGGCGACTCAGGCCTGTCCAGCCGGGGCAAGAA  
 H G E N E H N L Q G R I G G D S G L  
 GTTTGCCAGTGCTCTGAGCAAGTTCGTGGAGGAGCAGAACCTGAAGGACCTG  
 S S R G K K F A S A L S K F V E E  
 CGTGTGGACCAGCCAGCTGAAGAGCACCATCCAGACGGCCGAGGCGCTGCGC 1300  
 Q N L K D L R V W T S Q L K S T I  
 GGCTGCCCTACGAGCAGTGGAAGGCGCTCAATGAGATCGACGCGGGCGTCTG  
 Q T A E A L R L P Y E Q W K A L N E  
 TGAGGAGCTGACCTACGAGGAGATCAGGACACCTACCCTGAGGAGTATGCG 1404  
 I D A G V C E E L T Y E E I R D T  
 CTGCGGGAGCAGGACAAGTACTATTACCGCTACCCACCGGGGAGTCCTACC

FIGURE 2B

4/13

Y P E E Y A L R E Q D K Y Y Y R Y  
 AGGACCTGGTCCAGCGCTTGGAGCCAGTGATCATGGAGCTGGAGCGGCAGGA  
 P T G E S Y Q D L V Q R L E P V I M  
 GAATGTGCTGGTCATCTGCCACCAGGCCGTCCTGCGCTGCCTGCTTGCCTAC 1508  
 E L E R Q E N V L V I C H Q A V L  
 TTCCTGGATAAGAGTGCAGAGGAGATGCCCTACCTGAAATGCCCTCTTCACA  
 R C L L A Y F L D K S A E E M P Y  
 CCGTCCTGAAACTGACGCTGTGCTTATGGCTGCCGTGTGGAATCCATCTA 1612  
 L K C P L H T V L K L T P V A Y G C  
 CCTGAACGTGGAGTCCGTCTGCACACACCGGGAGAGGTCAGAGGATGCAAAG  
 R V E S I Y L N V E S V C T H R E  
 AAGGGACCTAACCCGCTCATGAGACGCAATAGTGTACCCCGCTAGCCAGCC  
 R S E D A K G P N P L M R R N S  
 CCGAACCCACCAAAAAGCCTCGCATCAACAGCTTTGAGGAGCATGTGGCCTC 1716  
 V T P L A S P E P T K K P R I N S F  
 CACCTCGGCCGCCCTGCCAGCTGCCTGCCCCCGGAGGTGCCACGCAGCTG  
 E E H V A S T S A A L P S C L P P  
 CCTGGACAAAACATGAAAGGCTCCCGGAGCAGCGCTGACTCCTCCAGGAAAC 1820  
 E V P T Q L P G Q N M K G S R S S  
 ACTGAGGCAGACGTGTGCGTTCCATTCCATTTCCATTTCTGCAGCTTAGCTT  
 A D S S R K H \*  
 GTGTCTTGCCTCCGCCCCGAGGCAAAACGTATCCTGAGGACTTCTTCCGGAG 2132  
 AGGGTGGGTGGAGCAGCGGGGAGCCTTGGCCGAAGAGAACCATGCTTGGC  
 ACCGTCTGTGTCCCCTCGGCCGCTGGACACCAGAAAGCCACGTGGGTCCCTG 2236  
 GCGCCCTGCCTTTAGCCGTGGGGCCCTCACCTCCACCTCTGGGTTTCTAGG  
 AATGTCCAGCCTCGGAGACCTTCACAAAGCCTTGGGAGGGTGATGAGTGCTG 2340  
 GTCCTGACAAGAGGCCGCTGGGGACACTGTGCTGTTTTGTTTCGTTTCTGTG  
 ATCTCCCGGCACGTTTGGAGCTGGGAAGACCACACTGGTGGCAGAATCCTAA 2444  
 AATTAAAGGAGGCAGGCTCCTAGTTGCTGAAAGTTAAGGAATGTGTAAAACC  
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 AGACGCAAGTTGGCTGGGTGGTCCACACCCTGGCTCTCCTGCAGGTCCACAC 2652  
 ACCTTCCAGGCCTGTGGCTGCCCTCCAAAGATGTGCAAGGGCAGGCTGGCTGC  
 ACGGGGAGAGGGAAGTATTTTGGCCGAAATATGAGAACTGGGGCCTCCTGCTC 2756  
 CCAGGGAGCTCCAGGGCCCCCTCTCTCTCCACCTGGACTTGGGGGGAAGTG  
 AGAAACACTTTCTCTGGAGCTGCTGGCTTTTGCACCTTTTGTGATGGCAGAAGT 2860  
 GTGACCTGAGAGTCCACCTTCTCTTCAGGAACGTAGATGTTGGGGTGTCTT  
 GCCCTGGGGGGCTTGGAACCTCTGAAGGTGGGGAGCGGAACACCTGGCATCC 2964  
 TTCCCCAGCACTTGCATTACCGTCCCTGCTCTTCCAGGTGGGGACCCGGA  
 TT

FIGURE 2B continued

FIGURE 3

PRG1	1	-----MPL	ELTQ	SRVQK	IWV	P	VDHRPS	L	--PR
hum. liver	1	-----MSPE	MGELTQ	TRLQK	IW	P	PHSSGSSRL	QRR	
bov. brain	1	SEGVEGR	RASRGK	MPL	ELTQ	SRVQK	IW	P	VDHRPS
bov. heart	1	-----MSGN	PASSE	Q	NN	SYETKA	SLR	ISEK	--KC
PRG1	26	SCG	-PKL	TNSPT	-V	I	V	M	V
hum. liver	32	GSS	I	PQFT	NSPT	M	I	-M	V
bov. brain	39	TCG	-PKL	TNSPT	-V	I	V	M	V
bov. heart	30	SWA	-SYM	TNSPT	-L	I	V	M	I
PRG1	65	PTK	V	F	N	V	G	E	Y
hum. liver	72	PTK	V	F	N	L	G	Q	Y
bov. brain	78	PTK	V	F	N	L	G	E	Y
bov. heart	69	PTK	V	F	N	L	G	V	Y
PRG1	106	ALR	D	V	K	S	Y	L	A
hum. liver	112	ALR	D	V	K	S	Y	L	A
bov. brain	119	ALR	D	V	K	S	Y	L	A
bov. heart	110	ALR	D	V	K	S	Y	L	A
PRG1	147	K	A	F	F	I	E	S	V
hum. liver	153	K	V	F	F	I	E	S	V
bov. brain	160	K	V	F	F	I	E	S	V
bov. heart	151	K	V	F	F	I	E	S	V
PRG1	187	F	M	K	R	I	S	C	Y
hum. liver	193	F	M	K	R	I	S	C	Y
bov. brain	201	F	M	K	R	I	S	C	Y
bov. heart	191	F	M	K	R	I	S	C	Y
PRG1	228	Q	D	H	I	Q	S	R	I
hum. liver	233	Q	D	H	I	Q	S	R	I
bov. brain	242	Q	D	H	I	Q	S	R	I
bov. heart	232	Q	D	H	I	Q	S	R	I



6 / 13

FIGURE 3 continued

[illegible]

7/13

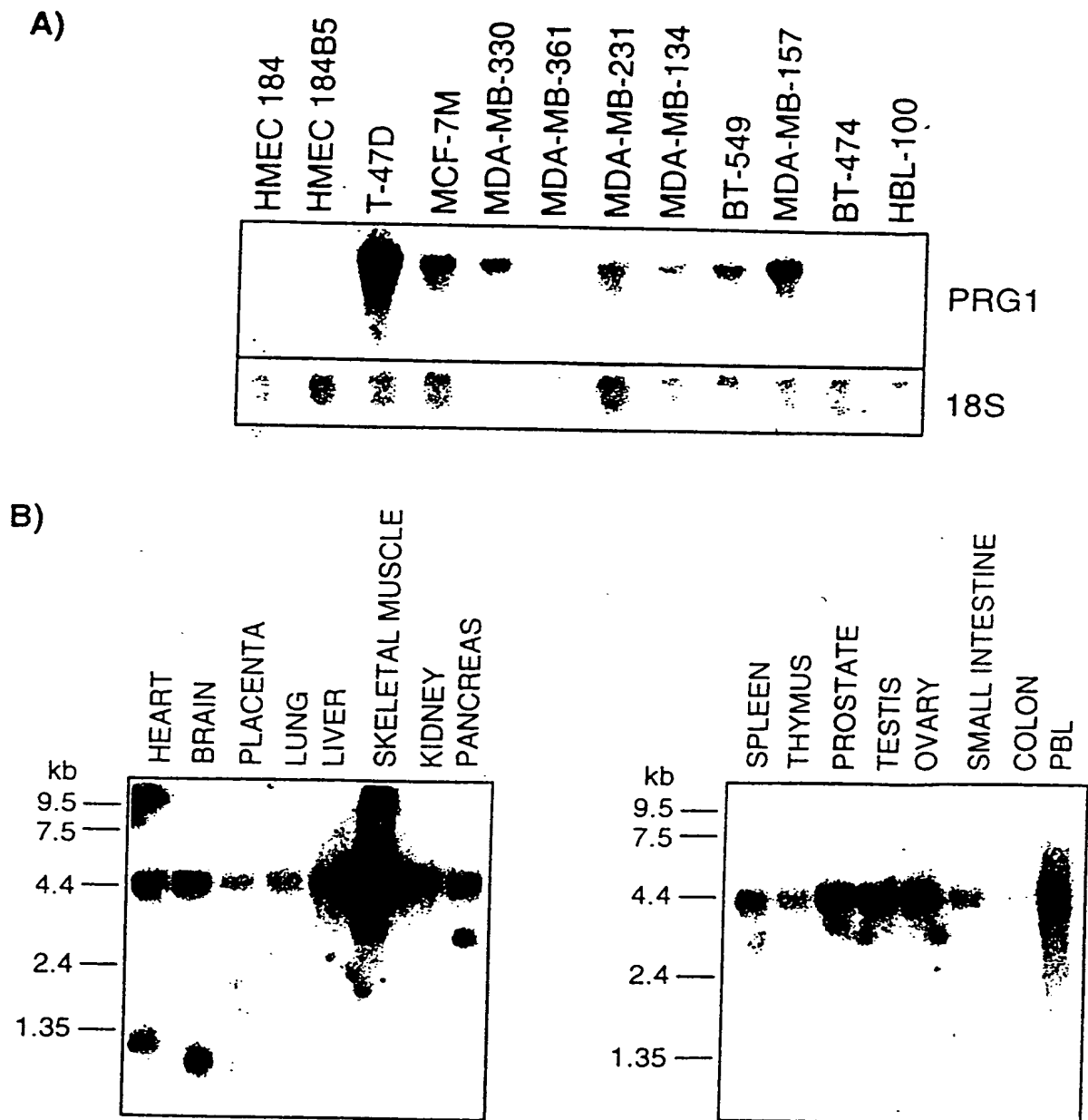
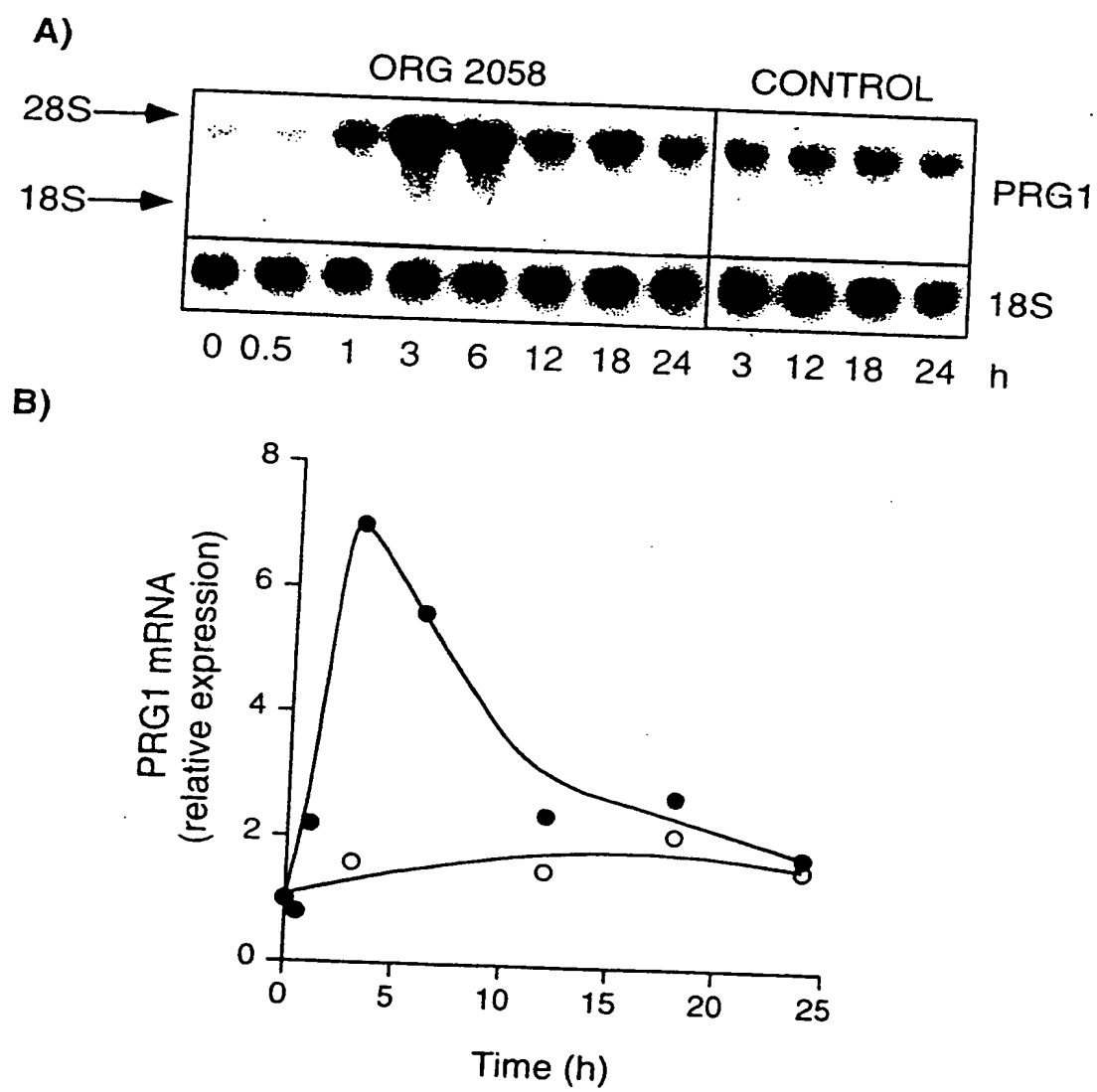


FIGURE 4

8/13

FIGURE 5



9/13

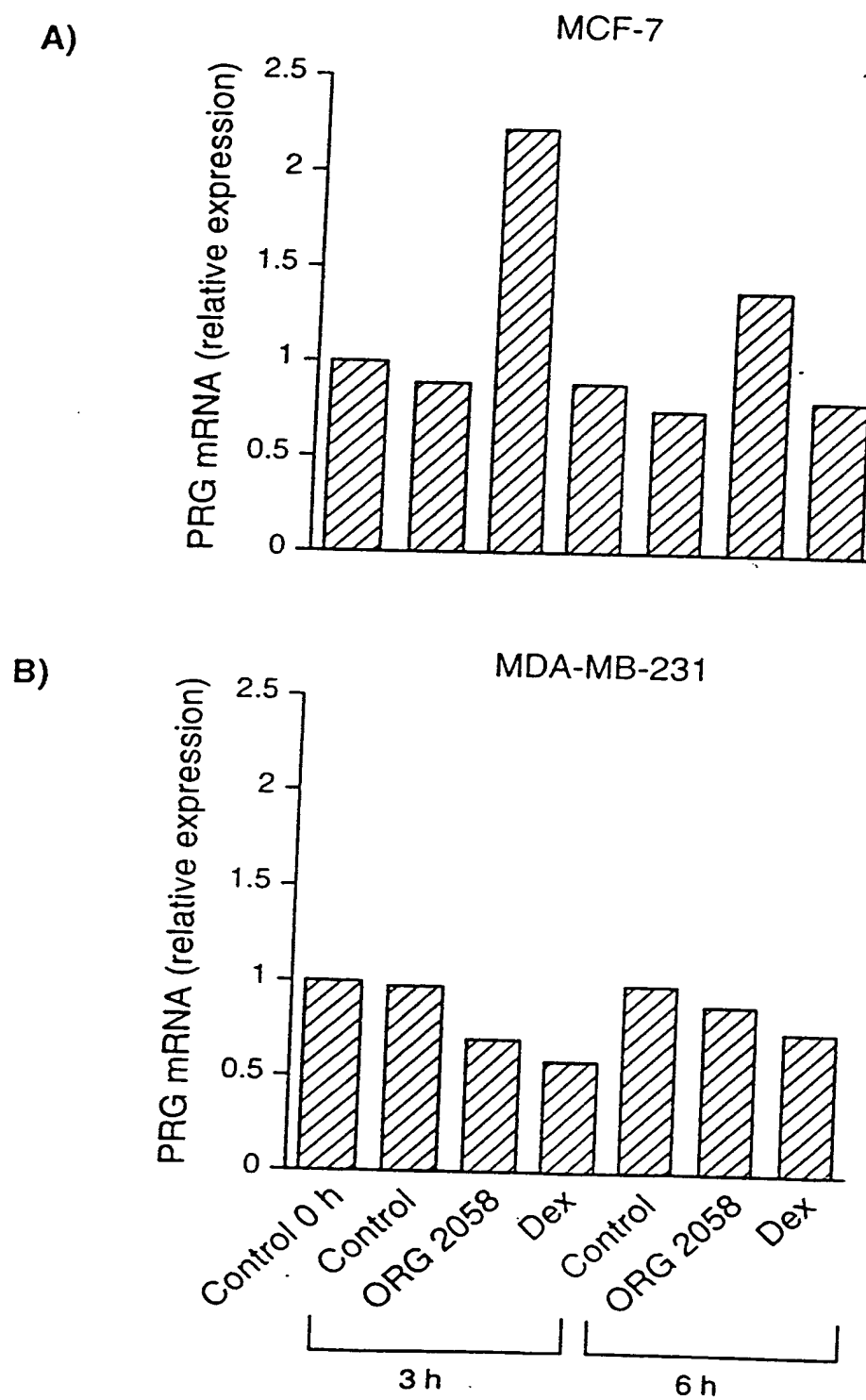


FIGURE 6

10/13

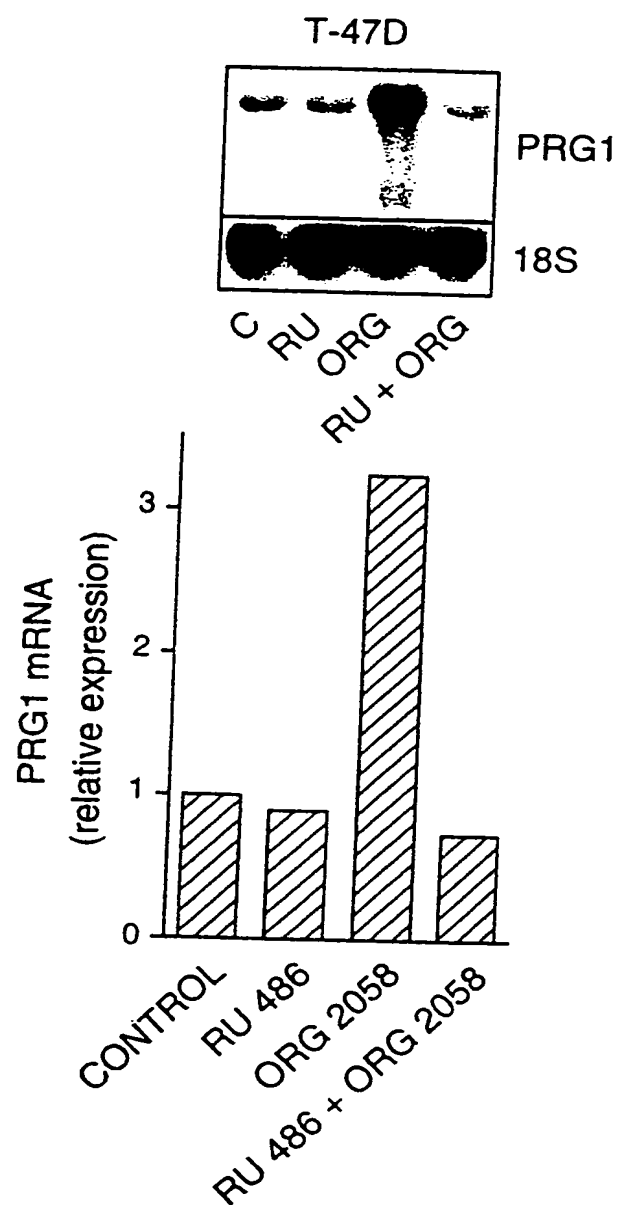
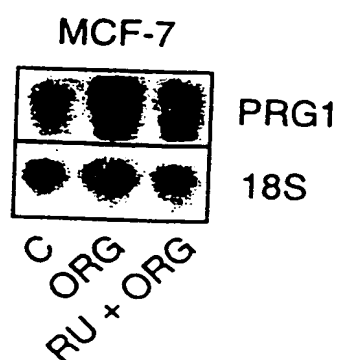
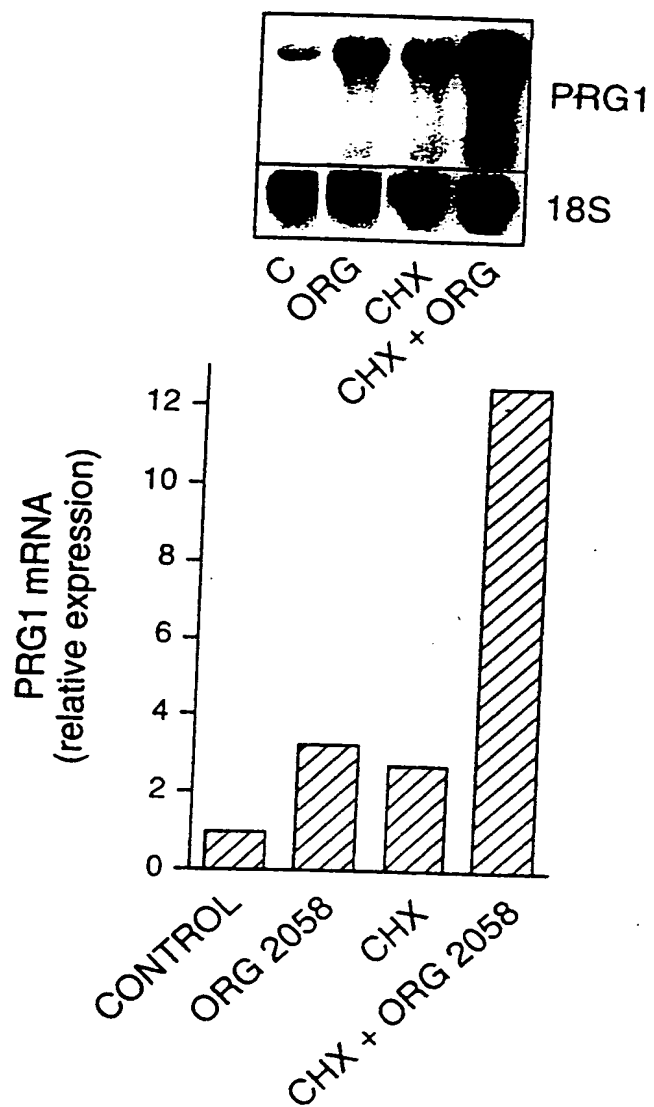
**A)****B)**

FIGURE 7

11/13

**A)**



**B)**

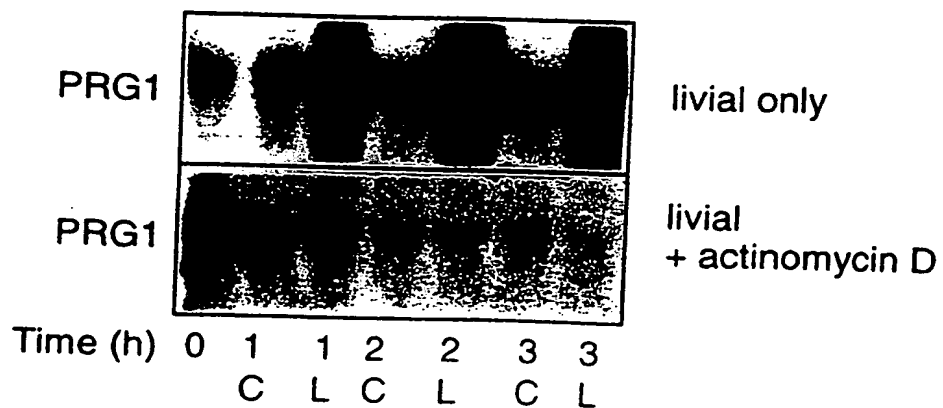


FIGURE 8

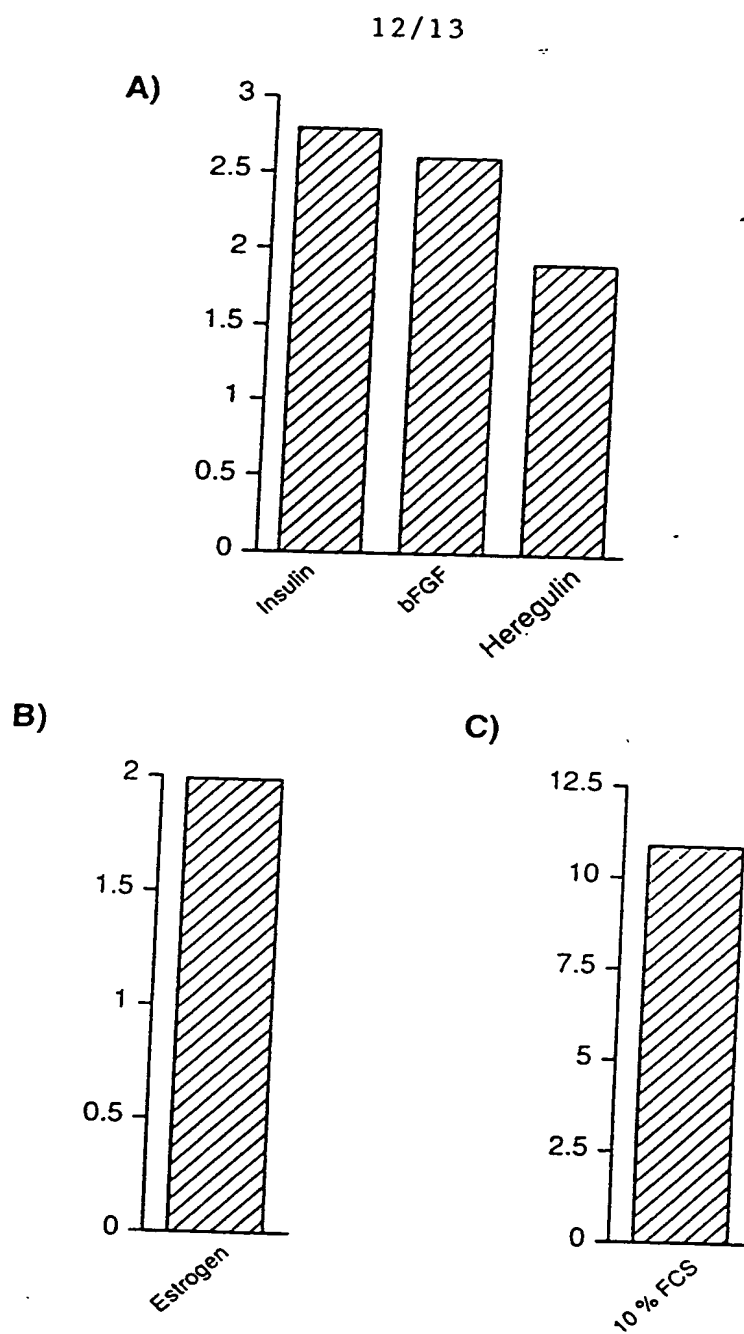
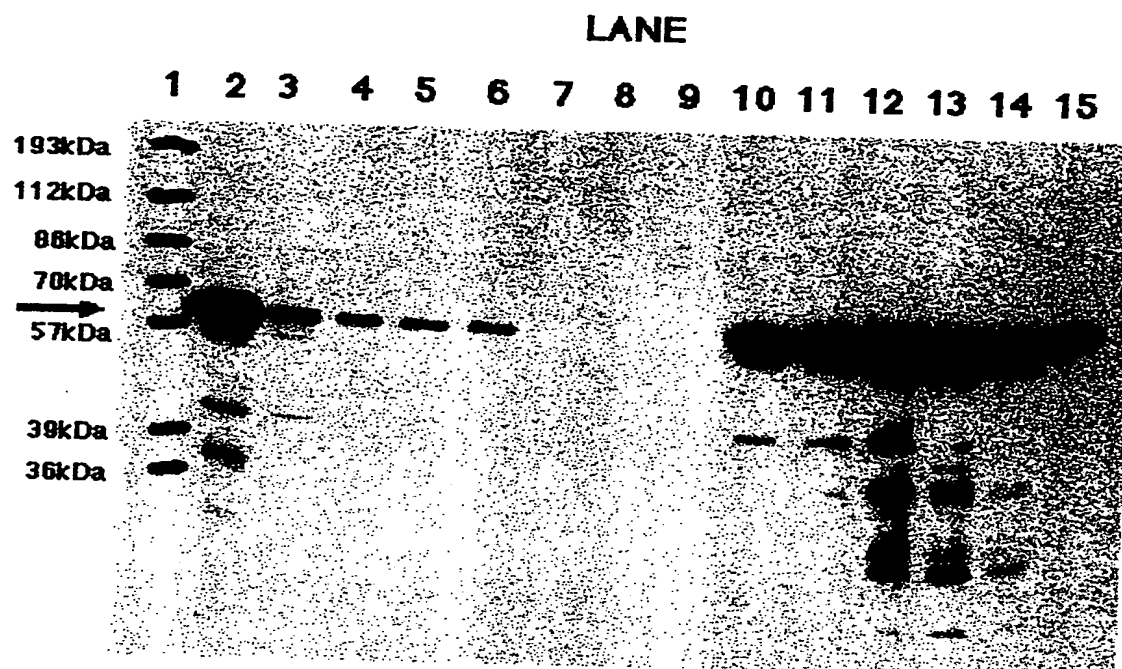


FIGURE 9

13/13

FIGURE 10





## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00669

**A. CLASSIFICATION OF SUBJECT MATTER**Int Cl<sup>6</sup>: C12N 15/55, C12N 15/54, C12N 9/12, C12N 9/16 C07K 16/40

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

See electronic database box below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
See electronic database box belowElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Chem Abs sequence search, amino acid sequence - MPLELTQSRVQ/EALRLPYEQ/RSSADSSRKH  
WPAT, JAPIO - Keywords - Progestin () regulat: () gene or PRG1**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Ventura, F. et al, "Cloning and Expression of a catalytic core bovine brain 6-Phosphofructo-2-Kinase/Fructose-2,6-biphophatase"  <u>Biochemical and Biophysical Research Communications</u> volume 209, Number 3, April 1995, pages 1140-1148.  See entire document	

☐ Further documents are listed in the continuation of Box C☐ See patent family annex

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

7 November 1996

Date of mailing of the international search report

8 JAN 1997

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## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00669

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1 and 2 partially  
because they relate to subject matter not required to be searched by this Authority, namely:  
sequences which are 80% and 90% homologous to the figures defined by these claims
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims 1 to 23 are directed to the DNA encoding PRG1, PRG1 itself, antibodies to it and assays and methods involving this polypeptide. The special technical feature, common novel feature or inventive concept resides in the PRG1 enzyme.
2. Claim 24 is directed to agonist and/or antagonist compounds which are identified as such by the methods of claims 20 to 23. This claim is a claim to these compounds per se which are not the product of these methods and as such does not incorporate the above stated special technical feature.
1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1 to 23

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.